

**p53 MUTATIONS AS A MARKER OF MALIGNANCY
IN BREAST AND BLADDER CANCER**

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DECLARATION

I declare that the thesis has been composed by myself and that the work contained in it has been undertaken by myself except where indicated and acknowledged.

H.A.Phillips

22/9/98

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ABBREVIATIONS

CEA	Carcino-embryonic Antigen
CIS	Carcinoma in situ
CDI	Carbodiimide
CMC	Chemical Mismatch Cleavage
DCIS	Ductal Carcinoma in situ
DDGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
IHC	Immunohistochemistry
LCIS	Lobular Carcinoma in situ
NAF	Nipple Aspirate Fluid
PCR	Polymerase Chain Reaction
PSA	Prostatic Specific Antigen
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
SSCP	Single Stranded Conformational Polymorphism

Abbreviations for chemicals and reagents are indicated in Materials and Methods (Chapter 2).

ABSTRACT

Mutations of the p53 gene are common in a wide variety of human malignancies. Detection of such mutations opens up the possibility of their use as a marker for the presence of malignancy, with potential applications in screening, diagnosis and follow up. This project investigated this potential. Single Stranded Conformational Polymorphism (SSCP), a PCR based gel electrophoresis technique, was used to screen for p53 mutations in bladder washing samples from patients with bladder cancer and breast fluid samples obtained by nipple aspiration from patients with breast cancer. Exons 5-8 of the p53 gene which contain over 80% of reported mutations were examined individually.

The SSCP assay was optimised using DNA extracted from 6 cancer cell lines (2 with normal p53 and the others each with a mutation in 1 of exons 5,6,7 or 8) using 6 sets of gel conditions. Each mutant-carrying cell line could be identified by an unambiguously abnormal SSCP pattern under at least 3 sets of gel conditions. Dilutions of each of the mutant cell lines were made with wild type cells to determine the sensitivity of SSCP to detect the mutation in DNA extracted from the mixture of cells. (This was of fundamental importance as the clinical samples studied would inevitably contain such a mixture). SSCP was performed for each dilution series using gel conditions able to demonstrate that particular mutation. The sensitivity in detecting mutations varied (range 1-30%) and was dependent on the conditions used. Using optimal conditions for each mutation a distinct extra band corresponding to the mutation was discernable at the 1 in 100 level for exon 8 and at 1 in 20 for exons 5, 6

and 7. These results illustrate the importance of gel conditions in determining the sensitivity of SSCP in detecting mutations in mixed cell populations. Because optimal detection conditions varied between mutations, SSCP was performed under 3 sets of gel conditions for each exon when screening clinical samples.

Bladder washing samples (31) were collected from patients (27) with bladder cancer. An abnormal additional SSCP band was detected in 5 samples from 5 different patients suggesting the presence of a p53 mutation. The mutation was in exon 8 in a single case and in exon 6 and 7 each on 2 occasions. In all 5 cases the same abnormal SSCP pattern was demonstrated in samples of the corresponding bladder tumour. In 3 cases the presence of mutation was confirmed by direct DNA sequencing, however in the 2 samples with exon 7 abnormalities it was not possible to obtain a reamplified PCR product for sequencing. In 1 case bladder washings were available from the same patient on 2 separate occasions with one washing demonstrating a mutation and the other not. In 2 further cases a mutation was demonstrated in the bladder tumour but not in the corresponding washing.

Breast ductal fluids were obtained by nipple aspiration from 21 female patients with histologically confirmed breast carcinoma. Corresponding breast tumour samples were also obtained. In view of the small amount of fluid available DNA extracted from excised tumour was screened for p53 mutations before studying the corresponding fluid samples. Of the 21 tumours examined 4 were found to have an abnormal SSCP pattern. The putative mutation was characterised in each case by direct sequencing. However it was not possible to demonstrate the mutation in any of the

four fluid samples from the breasts of these women. The problems encountered included the small amount of DNA extracted from the fluid samples and the demonstration that breast fluid contains a factor that inhibits PCR amplification.

In conclusion the sensitivity of SSCP to detect mutations in a mixed cell population is defined. It is shown that p53 mutations can be detected and characterised in DNA extracted from bladder washing samples from patients with bladder cancer, but for clinical applicability sensitivity needs to be improved. It has not been possible to demonstrate p53 mutations in breast ductal fluid. However further study of molecular abnormalities as disease markers is warranted in both bladder and breast cancer.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Malignant disease is a major cause of illness and death throughout the world. Whilst major advances have transformed the prognosis for patients with a variety of the less common malignancies such as leukaemia, lymphoma and testicular cancer, there has been little or only modest improvement in the cure rates for most of the common solid cancers in the last 30 years (Sikora et al 1995). It is hoped that improved understanding of the molecular pathology of malignant diseases can be translated into advances in treatment (Cortner and Vande Woude 1997). Furthermore diagnosis of primary or recurrent disease at an earlier stage than is currently possible may have prognostic benefit.

Mutations in specific oncogenes and tumour suppressor genes are implicated in the genesis and development of many cancers (Perkins and Stern 1997). The advent of polymerase chain reaction (PCR) technology enables the amplification of genetic material in quantities sufficient for sophisticated analysis (Eeles et al 1992). There is therefore potential for developing molecular biological techniques to aid in the diagnosis or monitoring of cancer, using the detection of mutations as a biochemical marker of disease. Cells exfoliated by tumours into body cavities and body fluids are an obvious and theoretically attractive medium for investigating the potential of such techniques.

This thesis describes a pilot study using mutations of the p53 gene, one of the genes most commonly subject to mutation in malignant disease

(Stratton 1992), as a molecular marker in 2 common diseases. These are breast cancer and bladder cancer, using nipple aspirate fluid and bladder washings respectively as the method of sampling exfoliated cells.

The introduction describes the background to the study. The clinical importance of both breast and bladder cancer are described. The importance of the p53 gene, it's relevance to the 2 diseases, and why aspects of its biology make p53 gene mutations potentially useful markers of cancer are explored. The biology of breast ductal fluid is reviewed in relationship to breast cancer as is the literature describing methods exploring the study of bladder cancer using material found in bladder washing and urine samples. Methods of detecting oncogene mutations are described and finally the formal aims of the study are set out.

1.2 Bladder Cancer

Bladder Cancer is a common disease giving rise to approximately 5000 deaths a year in the United Kingdom. The disease is more common in males than females and in caucasians than blacks (Keane et al 1995). Histologically the majority of bladder cancers in the Western developed world are transitional cell carcinomas with squamous cell carcinomas accounting for approximately 5% and adenocarcinomas less than 5% (Cotran et al 1989a). However in Egypt and parts of Central Africa, where Schistosomal infection is endemic, up to 80% of bladder cancers are squamous cell in origin relating to the irritative affects of chronic schistosomal infection on the bladder mucosa (Keane et al 1995). In the West, whilst bladder cancer can be related to chronic infection, certain occupational exposures (for example to organic amines and aluminium)

and drugs such as cyclophosphamide and phenacetin (Scher et al 1997), it can be regarded primarily as a smoking related illness (Murphy 1989).

Bladder cancer can be divided into superficial and invasive disease. In superficial disease the tumour is confined to the mucosa, submucosa or lamina propria of the bladder and the predominant clinical problem is of establishing local control of the disease because of the tendency for tumour recurrence and multifocality; these tumours rarely metastasise (Keane et al 1995, Scher et al 1997). The mainstay of treatment is transurethral resection with intravesical chemotherapy and intravesical immunotherapy having an adjunctive role in some clinical situations (Keane et al 1995, Newling et al 1995). The optimal treatment depends on the stage and histological grade of the tumour. Recurrence rates are as high as 50-80% but disease progression to muscle invasive disease occurs more rarely, in approximately 10-25% of patients (Keane et al 1995, Scher et al 1997). Muscle invasive disease is defined by the presence of tumour invading into the bladder's muscular wall or more deeply. In muscle invasive disease attempts at local tumour control are still important though in up to 50% of patients the natural history is predominated by the development of metastatic disease and ultimately death (Keane et al 1995). Treatment of the primary tumour involves either radical surgery by cystectomy and urinary diversion or external beam megavoltage radiotherapy with salvage cystectomy for locally recurrent or persistent disease. Both options have very similar results (Keane et al 1995). The treatment modality is thus determined by factors such as the age and general condition of the patient, patient choice, the expertise available and the philosophy of the doctors involved. The use of neoadjuvant and adjuvant chemotherapy has been extensively studied and the role of both

remains controversial (Keane et al 1995, Newling et al 1995, Scher et al 1997). In the United Kingdom chemotherapy in these settings is not widely used because of the lack of data supporting its use from good quality clinical trials. However the treatment of clinically apparent metastatic disease with platinum based combination chemotherapy offers the prospect of useful objective response rates (upwards of 40-60%) and worthwhile palliation of symptoms, but little prospect of cure (Scher et al 1997).

The risk of developing metastatic disease relates directly to the stage and grade of the tumour and therefore detection of the disease at an earlier time point in its natural history offers enhanced prospect of cure (Newling et al 1995). In the absence of curative treatment for metastatic disease methods of early disease detection are an appealing avenue of study. Furthermore as bladder cancer is a disease predominantly of elderly smokers with associated comorbidities, the development of methods useful in diagnosis or follow-up that could lessen the requirement for invasive procedures such as cystoscopy would be most useful.

1.3 Breast Cancer

Breast Cancer is the most common female malignancy and the leading cause of cancer related mortality in women in the United Kingdom (Keane et al 1990), though in Scotland lung cancer now causes more deaths, reflecting more an increase in the incidence of the latter disease in women rather than a major decline in breast cancer mortality (Scottish Health Statistics 1997). Breast cancers arise in the ductal and glandular structures of the breast and following the World Health Organisation

classification can be divided into noninvasive and invasive tumours (Azzopardi et al 1992). Noninvasive tumours are largely made up of Ductal Carcinoma in Situ (DCIS) and Lobular Carcinoma in Situ (LCIS). The natural history of such lesions is not precisely known, and therefore there is considerable uncertainty regarding optimal treatment (Harris et al 1997). Undoubtedly the results of mastectomy for DCIS are excellent in terms of survival, however for localised screen detected disease breast conserving techniques are under investigation with indications that treatment by wide local excision followed by radiotherapy, or in certain groups wide local excision alone is appropriate (Harris et al 1997). Following a diagnosis of lobular carcinoma in situ, it appears the risk of subsequently developing invasive cancer is about 9 times greater than that of the general population, both breasts being equally at risk from either ductal or lobular invasive disease (Harris et al 1997). Given this, LCIS is regarded as an indicator of increased breast cancer risk rather than a premalignant condition with the 2 main treatment options of either biopsy and careful observation or bilateral mastectomies reflecting this (Yarnold et al 1995, Harris et al 1997).

Invasive or infiltrating carcinomas of many types are described with by far the most common being invasive ductal carcinomas, which either in pure form or admixed with other types account for 75% of invasive breast cancers. Invasive lobular carcinomas and medullary carcinomas account for most of the remainder (Cotran et al 1989b, Yarnold 1995).

The etiology of breast cancer is complex, however as the breast is a structure that is subject to endocrine influences it comes as no surprise that hormones have a bearing on the development of breast cancer. The

risk of developing breast cancer increases with increasing length of reproductive life, with an early menarche and late menopause being adverse factors. Breast cancer is more common in the nulliparous than the multiparous whilst early age at first pregnancy and breast feeding have a protective effect (Cotran et al 1989b). The role of exogenous hormones is controversial but there would appear to be an increased risk with hormone replacement therapy (Harris et al 1997). Genetic factors are also important with inheritance of mutations in genes such as BRCA 1, BRCA 2 and p53 accounting for a small but distinct subset of breast cancers often with an early age of onset (Ford and Easton 1995).

The treatment of breast cancer is complex and rapidly evolving. The two major objectives of treatment are to obtain local disease control with due consideration to optimal cosmesis, and to maximise survival with appropriate systemic therapy. The treatment of local disease traditionally involves surgical removal of the tumour with either a wide local excision (for small tumours) followed by postoperative radiotherapy, or mastectomy with or without radiotherapy depending the clinical and pathological risk factors found for the individual patient (Yarnold 1995). The use of neoadjuvant or preoperative systemic therapy either with cytotoxic chemotherapy or endocrine treatments especially in patients with large primary tumours is the subject of ongoing evaluation (Cameron and Leonard 1995). The axillary lymph nodes are also treated by surgery or radiotherapy, though an appropriate surgical axillary node sample if pathologically clear of disease may obviate the need for further axillary treatment (Yarnold 1995).

From a meta-analysis of adjuvant systemic therapy it is clear that for most

patients the risk of metastatic disease and therefore risk of death from breast cancer is substantially reduced by treatment with either adjuvant hormonal therapy or polychemotherapy (Early Breast Cancer Trialists' 1992). What is less clear is whether combinations of hormonal and cytotoxic therapy have additional advantages for certain patient groups, and whether or not more intensive marrow ablative treatments have a role in those with adverse prognostic factors (Yarnold 1995, Harris et al 1997). Both these issues are currently being addressed in multicentre randomised clinical trials. There is also renewed interest in the influence of optimal local disease control on survival. Two recent publications suggest that reduction in loco-regional recurrence obtained by giving post-operative radiotherapy following mastectomy positively influences survival (Overgaard et al 1997, Ragaz et al 1997).

It is clear that despite advances in treatment the morbidity and mortality of breast cancer remain huge health care problems. It is also clear that patients presenting with early stage disease have a better prospect of long term survival (Yarnold 1995). It would therefore be hoped that by detecting disease at an earlier stage in its natural history the prospect of longterm survival may be improved. It was on this basis that breast cancer screening programmes were evaluated and subsequently widely introduced in many parts of the world. Randomised trials have shown an overall benefit for mammographic screening this being largely restricted to women in the 50-69 year old age group (Miller 1993, Yarnold 1995). Several issues remain unresolved, such as the optimum frequency of screening and the benefit to subjects outside the above age group. Several possible reasons for poor results in women aged 40 - 49 have been cited. Poor sensitivity of mammography in younger women with radiologically

dense breasts, possible differences in the biology of pre and postmenopausal breast cancers, and the relative failure of therapy in the younger age group may all be contributing factors (Miller 1993). Whatever the reasons it is relevant to consider other possible approaches to the earlier detection of breast cancer. In this regard the study of breast ductal fluid obtained by nipple aspiration is potentially interesting and the investigation of such fluid samples was one of the objectives of this study. The reasons for choosing nipple aspirate fluids as a vehicle for investigation and the relationship of such fluids to the biology of breast cancer are discussed in a subsequent section of the introduction (1.8).

1.4 p53: General Introduction

The p53 tumour suppressor gene and its protein product have been the subject of a vast amount of research work over the past 18 years, firstly because of their common involvement in the malignant process and secondly because of their biological complexity. Approximately 50% of human cancers carry structural changes in the gene, most commonly in the form of point mutations and in many others the effects of wild type p53 are perturbed by other mechanisms, making p53 aberrations an almost universal step in the development of human cancers (Hollstein et al 1991). The following is a brief overview of the structure and function of the gene and its product emphasising those features of most relevance to the present study, rather than an exhaustive review of the subject.

p53 was originally described in 1979 as a normal cellular protein that was able to bind to the large transforming antigen of the SV40 DNA virus (Lane and Crawford 1979, Linzer and Levine 1979). The gene is 20 kb long,

has 11 exons (Lamb and Crawford 1986) and is located on the short arm of chromosome 17, at 17p13.1 (Benchimol et al 1985, McBride et al 1986). The gene product is a 393 amino acid nuclear phosphoprotein 53 kd in molecular weight, hence the protein's name (Harris and Hollstein 1993). It is noteworthy that exon 1 is a noncoding sequence separated from exon 2 by a very long intron. The significance of this is unclear but may be a reflection of how the gene is regulated (Soussi et al 1990). 5 clusters or domains of amino acids are highly conserved between species implicating them as central to the protein's function and include the binding site of the large T antigen. Domains I, III, IV, and V are specified by parts of exons 2,5,7,and 8 respectively and domain II specified by parts of exons 4 and 5 (Soussi et al 1990). Domains II-V are found in the hydrophobic central part of the protein molecule and alterations in the composition of these domains through mutation can alter the structure and binding properties of the protein (Soussi et al 1990).

In human malignancies approximately 80% of mutations in p53 are missense point mutations which are frequently but not invariably associated with loss of heterozygosity at the gene locus (Harris and Hollstein 1993). The vast majority of p53 mutations in human cancers are somatic rather than germline. Inherited p53 mutations such as in the Li Fraumeni syndrome are exceedingly rare and characterised by a distinct clinical pattern of tumour types arising at a young age (Malkin et al 1990). It is now apparent that the majority of somatic point mutations detected are clustered in 4 hotspots coinciding with the 4 most highly conserved regions of the gene. These areas represented by codons 132-143 (exon 5), 174-179 (exon 5), 236-248 (exon 7), and 272-281(exon 8) accounted for 86% of mutations detected in an initial series from a variety of human cancers

(Nigro et al 1989). In a review of the mutational spectrum of p53 in human cancers 98% of described abnormalities were found between codons 110 and 307. Whilst the majority of studies looked only at exons 5-8, those that looked further suggest mutations outside this area are unusual (Hollstein et al 1991). Study of mutational spectra has revealed differences in patterns of mutation between different diseases and may well reflect the etiological influence of the exogenous and endogenous genotoxins implicated in the pathogenesis of those diseases (Hollstein et al 1991). This is most clearly illustrated by the effect of aflatoxin on the nature of p53 mutations in hepatocellular carcinoma (Harris 1996a).

The precise function or rather the full range of functions and relationships of p53 in normal tissues and in the malignant process is not as yet fully understood though an evolving model is emerging. p53 was originally thought to be an oncoprotein with transforming activity, however it has transpired that all the p53 constructs used in these early experiments contained p53 mutations and it was subsequently demonstrated that wild type p53 acts as a potent suppressor of transformation (Lane and Benchimol 1990), and it is now clear that p53 has antiproliferative and antitransforming activity, acting as an inhibitor of oncogenesis. p53 is therefore now regarded as a tumour suppressor gene and an eloquent model proposing p53 as the guardian of the genome has been described (Lane 1992). It is envisaged that p53 is a molecular policeman, accumulating when DNA is damaged, shutting off replication and allowing repair, or if this is not possible directing the cell to apoptosis. Cells without wild type p53 cannot carry out this arrest of the cell cycle; they are thus genetically less stable because division can occur without repair of the accumulating damage potentially leading to the production

of malignancy (Lane 1992). This model is depicted in Figure 1.1a (page 24). The importance of p53 in the process of apoptosis (cell suicide or programmed cell death) was emphasised by the subsequent demonstration that p53 is required for radiation induced apoptosis. Thus the loss of p53 can lead to inappropriate survival of cells following DNA damage and the potential for survival of cells that have undergone neoplastic transformation (Lowe et al 1993, Clarke et al 1993). This however is not the whole story and it is evident that whilst wild type p53 may be a tumour suppressor, at least some of the mutant forms as illustrated by the original work on p53's function have transforming effects more typical of an oncogene product. This is further confirmed by the demonstration that certain mutants exhibit gain of function, such as enhanced tumourigenicity and transactivation of the MDR1 (multiple drug resistance) gene promoter (Dittmer et al 1993). This taken together with the requirement for the integrity of the transactivation domain of the protein suggest that gain of function is dependent on the action of mutant p53 as an aberrant transcription factor (Lin et al 1995). It would appear therefore that wild type p53 is a tumour suppressor but at least some mutant forms of the protein are cancer promoting.

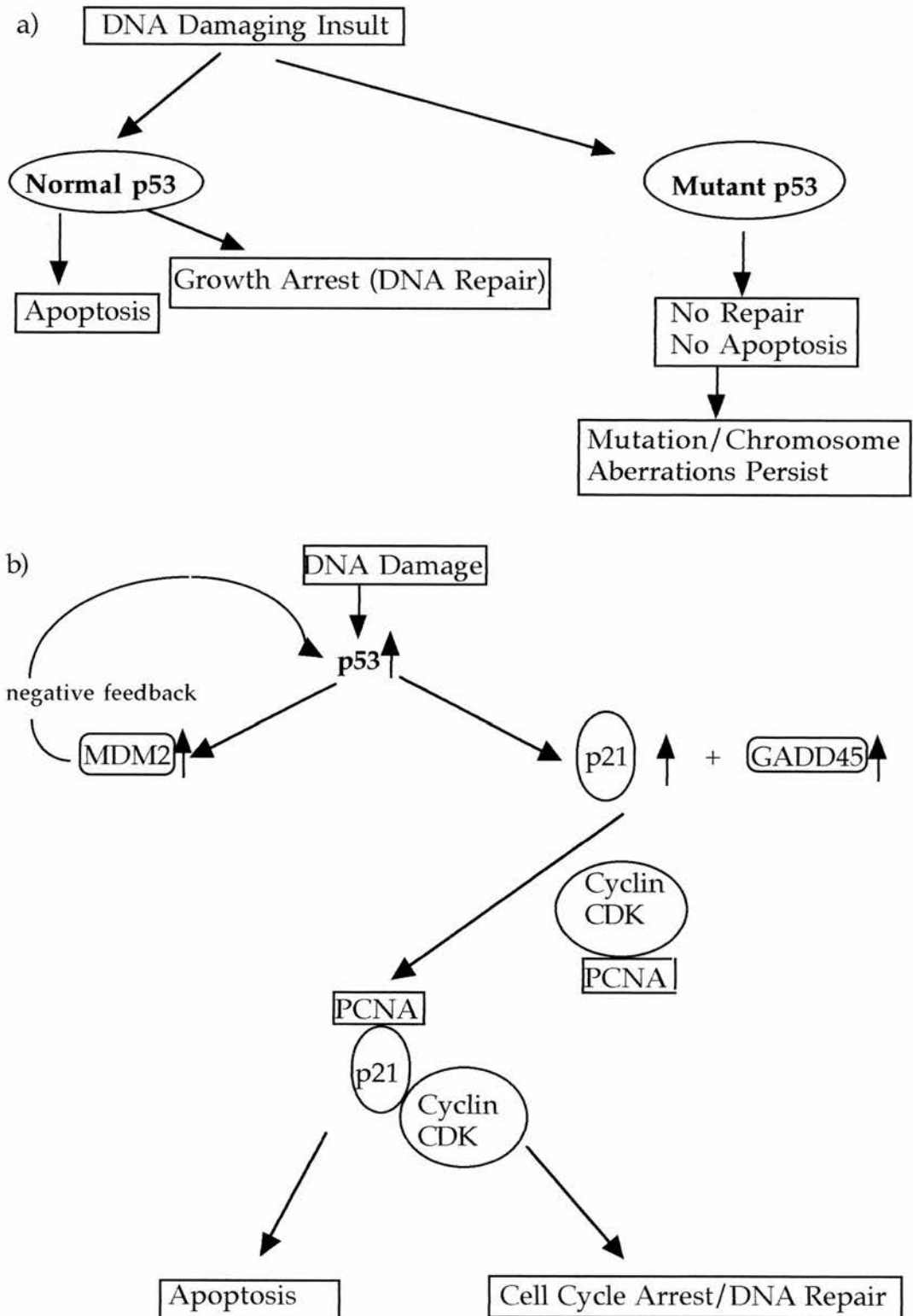
Before considering the mechanisms by which p53 exerts its effects in more detail it is appropriate to summarise what is known about the structure of the protein itself. It consists of 3 main regions. Firstly an N or amino terminal region of 75 amino acids is primarily involved in transcriptional activation. A central domain is largely involved in the sequence specific binding of the molecule to DNA (critical to wild type p53 function) and a carboxy or C terminal region contains nuclear localisation signals, sites recognising DNA damage and sites involved in oligomerisation of the

protein (Levine et al 1994, Harris 1996b). From the above it could be proposed that p53 transactivates the genes adjacent to sites of sequence specific DNA binding (Vogelstein and Kinzler 1992). It would appear that such binding is most efficient when p53 forms tetramers and that the formation of mixed tetramers between wild type and mutant forms of p53 is responsible for the dominant negative effects demonstrated by some mutant forms of p53 (Wang et al 1994, Milner 1995). The structure of p53 and the relationship of this to its function is illustrated in Figure 1.2 (page 25).

The demonstration that the central core of the p53 protein contains the sequence specific DNA binding domain (Pavletich et al 1993), the elucidation of the central core's crystal structure and how this relates to DNA binding (Cho et al 1994) has allowed insight into how mutations of the gene exert their effect. The majority of p53 mutations are missense mutations in the highly conserved areas of the gene, the very areas coding for the central core that is critical for site specific DNA binding and hence gene transactivation, and subsequently the downstream effects of the protein. Mutations in this region can be divided into 2 broad groups, firstly DNA contact mutants in which critical DNA contact residues are aberrant preventing binding and secondly structural mutants in which DNA binding is lost through the mutation leading to an abnormal protein conformation (Rolley et al 1995).

p53 mediates its effects in 3 main ways, firstly it may be directly involved as an inhibitor in the process of DNA replication, secondly it acts as a transcription factor for some genes and lastly may repress transcription of others (by binding to TATA binding protein and thus inhibiting its

Figure 1.1
Role of p53 in Response to DNA Damage
(Adapted from Carson and Lois 1995)



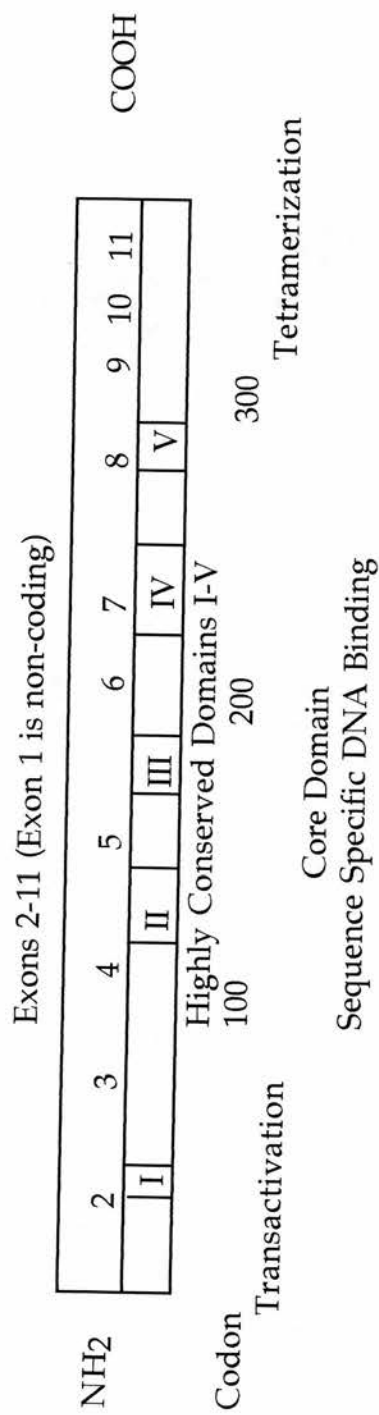


Figure 1.2: Diagrammatic representation of the p53 Gene, relating structure of the protein to coding sequence, and illustrating areas of high evolutionary conservation. (Adapted from Soussi et al 1990, Cho et al 1994)

function as transcription factor) (Barton 1995, Selivanova and Wiman 1995, Harris 1996b). Examples of genes transactivated include GADD 45 (growth arrest on DNA damage) , p21 or WAF1, BAX and those repressed include Bcl2, IGF-IR and IGF-II, that is the transactivation of genes promoting growth arrest and apoptosis is enhanced and that of genes inhibiting apoptosis is repressed (Harris 1996b). A simple model of p53's response to DNA damage has been described in which GADD45 and p21 production are enhanced. These proteins associate with PCNA (proliferating cell nuclear antigen) and most CDKs (cyclin dependent kinases) factors important in DNA replication and cell cycling, promoting growth arrest and directing the cell towards DNA repair or apoptosis if repair is not possible (Carson and Lois 1995, Harris 1996b). Obviously this model is incomplete but it emphasises the main tumour supressing activities orchestrated by wild type p53 namely cell cycle control, DNA repair and apoptosis. This model is illustrated in Figure 1.1b (page 24).

Whilst, because of the nature of the current study the frequency and nature of p53 mutations has been emphasised it is important to stress that p53 can be inactivated by at least 2 other mechanisms. Firstly the transforming proteins of several DNA viruses including the large T antigen of SV 40 and the HPV 16/18 E6 protein can bind and sequester p53 preventing its normal function, possibly with the purpose of allowing progression to S phase thus allowing viral DNA replication (Selivanova and Wiman 1995). Secondly binding and sequestration can occur with certain nuclear proteins, predominant amongst these being MDM2 , a protein regulated by p53 itself and thus probably normally involved in a negative feedback control loop (Selivanova and Wiman 1995, Harris

1996b). Gene amplification of MDM2 and consequent abnormal sequestration of p53 has been shown to be important in the escape of some sarcomas from p53 mediated growth control (Oliner et al 1992).

It is thus apparent that p53 can be inactivated through mechanisms that demonstrate loss of function and/or gain of function, with the potential for some aberrant forms of the protein to exert dominant negative effects on wild type protein.

Most interest has focussed on the role of abnormal p53 in tumour development. However of potentially equal importance is the realisation that many anticancer therapies exert their effects through DNA damage and the induction of apoptosis as a result of the damage inflicted. Of relevance therefore are clinical and laboratory studies implicating p53 mutations in the resistance to a variety anticancer agents by the attenuation of apoptosis, and the consequent notion that p53 may be an interesting molecule to target therapeutically (Lowe 1995).

Much of the literature on p53 in human malignancy has focused on the correlation between p53 status and clinical, pathological and biological factors. It is relevant therefore to discuss briefly the methods employed in assessing p53 status. Two major approaches have been adopted. The first involves DNA analysis either by direct sequencing or more commonly by selective sequencing of only those samples identified as mutant by a mutation screening technique such as Single Stranded Conformational Polymorphism (SSCP) (Casey et al 1996). The more widely used approach has been to assess p53 status by immunohistochemistry (IHC) (Casey et al 1996). Normal p53 has a very short half life of only a few minutes

(Reihnsaus et al 1990) and is therefore not detectable by IHC. Most mutant forms of the protein have a prolonged half life allowing immunohistochemical detection (Hinds et al 1990, Iggo et al 1990), the implication being that if there staining is present there is mutant protein and thus a mutation in the gene (Varley et al 1991). There remains controversy as to the reliability of each of these approaches in detecting p53 mutations (Casey et al 1996, Sjogren et al 1996). DNA based studies have largely concentrated on the more conserved areas of the gene (exons 5-8) and may thus underestimate the frequency of mutations (Elledge 1996) . Furthermore methods such as SSCP will fail to detect 10% -30% of mutations (Prosser 1993). A number of methodological factors have a profound influence on immunohistochemical staining. These include the antibody used (Elledge et al 1994a) , whether the tissue is frozen or fixed (Bartek et al 1990b), and the method of tissue fixation (Fisher et al 1994). Such factors as well as the subjective nature of IHC scoring and the absence of a uniform definition of abnormality all contribute to the diversity of results reported (Elledge 1996). Furthermore it is apparent that certain mutations such as splice site mutations and premature stop codons may not give rise to the expression of any protein and therefore no staining (Casey et al 1996, Sjogren et al 1996). Additionally in some circumstances p53 can accumulate in the absence of detectable gene mutation with the biological significance of this remaining unclear (Casey et al 1996). Nevertheless IHC is widely available and easy to perform and consequently despite the above reservations has allowed elucidation of many facets of the role of p53 in malignant disease.

In summary p53 appears to have a pivotal role in the cell's response to genotoxic stress. It is commonly mutated in human cancers and if not is

frequently inactivated by other means. Most mutations are missense mutations concentrated in exons 5-8 of the gene, the nature of the mutations reflecting the selection advantage in terms of aberrant growth conferred by the mutation rather than total loss of protein. The frequency of mutations lend the detection of such mutations to investigation as potential molecular markers of malignancy. The localisation of the mutations found within the gene and their nature determine appropriate strategies for their detection, as is discussed later.

1.5 p53 in Bladder Cancer

It is likely that accumulation of several genetic abnormalities is involved in the development of bladder cancer (Linehan et al 1997). Whilst the series of events is not yet as clearly defined as in the pathway described for colon cancer (Fearon and Vogelstein 1990), it has been proposed that there are at least 2 distinct molecular pathways in bladder cancer. Chromosome 9 abnormalities appear to be important in the development of superficial tumours with low risk of progression to invasive disease and p53 mutations being implicated as an early event in the development of carcinoma in situ lesions with their higher propensity to progress (Spruck et al 1994).

An increasing body of work is accumulating regarding p53 mutations in bladder cancer. On the basis that loss of heterozygosity is common in the 17p region in bladder tumours (Olumi et al 1990) Sidransky and colleagues (1991) studied 18 bladder cancers and found 11 (61%) to contain p53 mutations. Subsequently in another small series it was reported that p53 mutations were common especially in invasive and high grade

tumours (Fujimoto et al 1992). In a larger series Esrig et al (1993) found 32/73 tumours to contain mutations most commonly in exon 8 where a hotspot was detected at codon 280. Mutations were found in the other exons (5-7) studied and a relationship was found between the site of the mutations and the intensity of immunohistochemical staining. Other sizeable series have confirmed p53 mutations to be frequent (Spruck et al 1993, Spruck et al 1994, Goto et al 1997). Whilst it is difficult to make an accurate estimate of the percentage of patients with bladder cancer whose tumours contain p53 mutations, an estimate of around 40-60% of patients with invasive disease seems reasonable, although lower incidences have been reported (Suzuki and Tamura 1993, Oyasu et al 1995).

Several studies have focussed on the molecular epidemiology of bladder cancer with special reference to the role of p53. As discussed earlier bladder cancer is predominantly a smoking related disease in this country, however other aetiological factors are important, for example Schistosomiasis is implicated in the endemic form of Squamous cell carcinoma of the bladder found in Egypt (Keane et al 1995). Endogenous (spontaneous) and exogenous or environmental mutagens are responsible for specific types of mutation , with transitions for example resulting from spontaneous deamination of 5-methylcytosine to thymidine and G:C to T:A transversions commonly induced by environmental carcinogens (Williamson et al 1994). The high frequency of such transversions found amongst p53 mutations in bladder cancers would suggest a major role for exogenous mutagens in bladder cancer (Williamson et al 1994). G:C to T:A transversions are common in lung cancer and whilst a high incidence has been described in smokers with bladder cancer as compared to nonsmokers (Uchida et al 1995) others

have not found this (Spruck et al 1993). Transitions are more common in Schistosomal bladder cancer and it has been proposed that nitric oxide released as part of the inflammatory response to infection in the bladder may be responsible for the resultant deamination of 5-methylcytosine (Warren et al 1995). The pattern of p53 mutations in urothelial tumours associated with phenacetin abuse is in keeping with the drug having a chronic irritative effect rather than causing promutagenic DNA lesions (Petersen et al 1993). It is also noteworthy that multiple p53 mutations (generally an uncommon event) have been reported in the bladder cancers of smokers (Spruck et al 1993) and arylamine dye workers (Taylor et al 1996) as well as in those associated with Schistosomiasis (Ramchurren et al 1995) and in arsenic related bladder cancers in the black foot disease area of Taiwan (Shibata et al 1994).

It is clear that p53 mutations (whether assessed by IHC or direct mutation detection) are more common in tumours of high grade rather than low grade and in invasive disease rather than superficial disease (Wright et al 1991, Fujimoto et al 1992, Esrig et al 1993, Furihata et al 1993, Miyamoto et al 1993). Furthermore mutations are more common in carcinoma in situ than in papillary superficial tumours. The presence of p53 immunohistochemical staining in greater than 20% of cells from carcinoma in situ samples has been shown in a small series to be strongly associated with disease progression and on multivariate analysis this appeared to be independent of other variables (Sarkis et al 1994). Likewise an association has been found between p53 staining and disease progression following transurethral resection for T1 bladder cancers (Sarkis et al 1993) and whilst this may be because p53 positive (or mutant) tumours tend to be of high grade (Thomas et al 1994) these data reflect

the biological aggressiveness associated with p53 aberrations.

Given the association between biological aggressiveness and p53 mutations it is reasonable to consider whether or not p53 has a role as a prognostic factor of outcome in bladder cancer. In an early series whilst p53 overexpression as detected by IHC was associated with poor survival this was not independent of known prognostic factors (Lipponen 1993a). A similar finding has been reported for mutations detected by PCR-SSCP (Vet et al 1994). However an influential report on a large series of 243 cases of bladder cancer treated by cystectomy showed that p53 accumulation detected by IHC correlated more strongly with disease recurrence and survival than either stage or grade (Esrig et al 1994). Additionally p53 overexpression assessed in 90 patients all treated with the same regimen of neoadjuvant chemotherapy was an independent adverse prognostic factor (Sarkis et al 1995). Further, more recent studies describe no independent prognostic value attributable to p53 status, or to value only in a subset of patients (Kuczyk et al 1995, Nakopoulou et al 1995, Glick et al 1996, Okuno et al 1996). Given the diversity of methods used to assess p53 and the heterogeneity of the patients both within and between cohorts, this is not surprising. Thus, whilst it appears p53 continues to show promise as a prognostic marker, further large studies of uniformly treated patients assessed by standard methods are required. Given the pivotal role of p53 in the cells response to DNA damage and the mechanisms of action that most non-surgical anticancer therapies display, further work to elucidate the predictive value of p53 status in relation to response to specific treatments is required, however preliminary data have not demonstrated a relationship between radiosensitivity and p53 status in bladder cancer (Ogura et al 1995). It is

also important that at some stage the prognostic and predictive effects of p53 status as assessed by immunohistochemistry and by sequence analysis are compared so that the significance of the widely recognised discordance between these methods can be addressed.

Where precisely the development of p53 mutations fits into the natural history of bladder tumours remains unclear. There appear to be at least 2 molecular pathways for bladder cancer development, with p53 mutations occurring early in the pathway associated with aggressive invasive disease (Spruck et al 1994). The fact that loss of heterozygosity at the p53 locus (Tamada et al 1994) and p53 mutations can occur before muscle invasion (Oyasu et al 1995, Schlechte et al 1997) as well as the recent demonstration that the acquisition of p53 mutations may precede the diagnosis of invasive disease by some months (Vet et al 1996) emphasises the potential for screening for p53 mutations as a useful way of identifying patients with a high risk of disease progression. The detection of identical mutations in multiple and recurrent tumours suggests a high but not 100% incidence of clonality in such tumours (Habuchi et al 1993a, Petersen et al 1993, Vet et al 1996, Goto et al 1997). This lends credence to a potential use of a known mutation for an individual patient as a marker for monitoring disease in follow up after treatment. Further the frequent presence of p53 mutations especially in high grade and invasive tumours makes the detection of such mutations a potential marker for the presence of disease. The question remains as to which exons should be studied in the present investigation. Several groups have studied exons 5-8 alone on the basis of the early work suggesting these areas harbour most mutations (Miyao et al 1993, Petersen et al 1993, Spruck et al 1993, Shibata et al 1994, Vet et al 1994, Schlechte et al 1997). Others have looked at

additional exons but found mutations only in 5-8, Sidransky et al (1991): exons 5-9, Lianes et al (1994): exons 2-9 and Cordon-Cardo et al (1994): exons 5-9. Others studying various combinations of exons 2-11 have found only 9 of 54 mutations to lie outside exons 5-8 namely 4 in exon 4, 3 in exon 10 and 1 each in exons 9 and 11 (Habuchi et al 1993b, Miyamoto et al 1993, Williamson et al 1994). Given the above it was decided to screen only exons 5-8 alone in the current study accepting that some mutations would be missed; this was felt acceptable as the prime objective of the study was to establish the principle that mutations could be indentified and characterised in bladder washings rather than carrying out a comprehensive investigation characterising every mutation present.

1.6 p53 in Breast Cancer

Initial pointers to the fact that p53 mutations may be involved in breast carcinoma came from the observation that 17p was a frequent site of loss of heterozygosity in the disease (Mackay et al 1988). Prosser et al (1990) were the first to demonstrate p53 mutations in breast cancer when they found 13% of 60 tumours to contain mutations in exons 5 or 6. Further work from the same group found 41 mutations in 136 breast cancers when studying exons 5-9 and estimated that up to 40% of breast tumours were likely to contain mutations if the whole gene was considered (Coles et al 1992).

There is debate as to whether immunohistochemistry (IHC) is a reliable marker of p53 mutation in breast cancer. Deng et al (1994) found IHC to be quite specific but not very sensitive in detecting mutations and whilst others (Lohman et al 1993, Schneider et al 1994) have found reasonable

correlation between the two, others report a significant disparity (MacGeogh et al 1993). Some of this inconsistency may be accounted for by methodological differences between series. Interestingly it has been shown that cytoplasmic staining of p53 detected by IHC can occur with normal p53, presumably the p53 being inactivated and accumulating by nuclear exclusion rather than by mutation (Moll et al 1992). A comprehensive study which compared the prognostic significance of p53 sequence abnormalities and IHC found sequence data to be of greater prognostic value, and documented the discrepancy rates between the 2 methods of assessing p53 status, and explored their significance (Sjogren et al 1996).

Reported incidence of p53 mutations vary between series from 16% to 40% (Andersen et al 1993, Dunn et al 1993, Merlo et al 1993, Sasa et al 1993, Tsuda et al 1993, Saitoh et al 1994). A rate of 40% of locally advanced breast cancers bearing mutations has been reported (Faille et al 1994) and as will be discussed below the frequency of mutations has been noted to vary between histological types of breast cancer (Marchetti et al 1993 a,b). Mutation rates of 13-22% have been reported from CIS (Harris 1992, Elledge et al 1993). Direct comparison is again difficult between series because of methodological differences and differences in the part of the gene studied. The majority of studies have been confined to exons 5-8 or 5-9 and whilst mutations do occur outside these areas they are comparatively rare (Casey et al 1996, Sjogren et al 1996). It is perhaps reasonable to estimate that over 80% of p53 mutations in breast cancer are found in exons 5-8.

It is clear that breast tumours commonly accumulate abnormalities in

several genes during carcinogenesis but unravelling the genetic events in breast cancer is hampered by difficulty in studying early and preneoplastic lesions (Walker and Varley 1993). The nature, number and sequence of events is not clear in breast cancer nor is the precise place of p53 in scheme. It was believed that acquired or somatic p53 mutations were found solely in malignant lesions (Bartek et al 1990 b, Allred et al 1993 b, Eriksson et al 1994). However a number of immunohistochemical studies imply the presence of accumulated p53 in occasional benign tumours and epithelial hyperplasias of the breast (Heyderman and Dagg 1991, Koutselini et al 1991, Schmitt et al 1995). The significance of this p53 staining is uncertain, as is whether p53 positivity in this setting equates with the presence of mutation, though a recent report has confirmed the presence of mutations in a small number of benign breast lesions of varied histological type (Millikan et al 1995). This having been said it appears that p53 overexpression and p53 mutations are rare events in normal breast and in benign lesions. There is mounting evidence that p53 mutations occur quite frequently in DCIS, and that mutations are more common in high grade or comedo type DCIS (i.e. those lesions that histologically have the highest risk of progressing to invasive cancers) (Bartek 1990b, Poller et al 1993, Eriksson et al 1994, O'Malley et al 1994). It has been demonstrated in tumours having both invasive and in-situ components that the immunohistochemical staining pattern and intensity was the same in all cytologically malignant cells, and furthermore that the presence of the same mutation can be confirmed in both components (Davidoff et al 1991). These observations are consistent with p53 being involved in the early stages of breast cancer development (Harris 1992).

Both immunohistochemical studies and gene sequencing data have suggested that the frequency of p53 aberrations differ between histological types of breast cancer. Mutations are more common in invasive ductal carcinomas than in lobular cancers (Poller et al 1992, Marchetti et al 1993a,b) and mutations are rare in the well-differentiated special types of breast carcinoma with favourable prognosis (Marchetti et al 1993a,b, Rosen et al 1995). The exception to this would appear to be medullary carcinomas which although demonstrating some features of poor differentiation have a favourable prognosis but frequently display p53 mutations (Marchetti et al 1993a,b, Rosen et al 1995). It would appear important therefore to consider the proportions of tumours of various histological types when comparing series (Domagala et al 1993) especially if p53 status is being related to prognosis.

The nature and locations of p53 mutations found in a particular malignant disease may reflect the nature of causative mutagenic insults. In reviewing the spectrum of mutations in breast cancer Biggs et al (1993) found a higher incidence of G to T transversions at CpG dinucleotides when compared to colon cancer with a hotspot at codon 157. A similar pattern is seen in lung cancer and is more in keeping with the effect of an as yet unidentified exogenous mutagenic agent rather than random endogenous mutational events (Biggs et al 1993). The observation of different patterns of mutation in specific populations of patients and the apparent high incidence of p53 mutations in Japan, a country with a low incidence of breast cancer, emphasise that molecular approaches to epidemiology may advance the understanding of the biology of the disease (Blasyk et al 1994, Hartmann et al 1996).

With regard to bilateral breast cancers, whilst an initial immunohistochemical study suggested that somatic p53 mutations did not play a major role in the pathogenesis of bilateral disease (Ackerman et al 1995), a subsequent study demonstrated a significantly higher incidence of p53 mutations in bilateral compared to unilateral cancers, implying the possibility of biological or aetiological differences (Kinoshita et al 1995).

Given the rarity of breast cancer in males the series investigating p53 in relation to this disease are understandably small. The findings are however consistent with those reported for the disease in women. Immunohistochemical studies have shown p53 positivity in 29% and 54% of cases studied (Bruce et al 1996, Joshi et al 1996) and point mutations have been detected in 12 of 29 (41%) tumours (Anelli et al 1995) with a suggestion that p53 status may be of prognostic importance (Anelli et al 1995).

Somatic mutations in breast cancer are clearly common events. It is thus relevant to consider whether inherited or germline mutations have an important role in the disease. Germline p53 mutations are the underlying defect giving rise to the Li-Fraumeni familial cancer syndrome, one feature of which is a high incidence of breast cancer (Malkin et al 1990). It appears however that germline mutations are rare in non-Li-Fraumeni familial breast cancer (Prosser et al 1991, Warren et al 1992, Preudhomme et al 1993) with the majority of multiple case families attributable to BRCA1 and BRCA2 mutations (Ford and Easton 1995). Likewise studies on patients who have developed breast cancer at a young age (Borresen et al 1992, Sidransky et al 1992) or who have bilateral disease (Liderau et al 1992) show that inherited p53 mutations are rare events in these settings.

Abnormal p53 (whether assessed by immunohistochemistry or mutation analysis) is associated with a number of clinical, pathological and biological adverse prognostic factors in breast cancer. Abnormal p53 is a common feature of locally advanced (Faille et al 1994) and inflammatory breast cancers (Riou et al 1993), although when pooling the results of several studies there does not appear to be a clear relationship between either tumour size and p53 status (Elledge et al 1993) or axillary lymph node involvement and p53 status (Harris 1992). p53 abnormalities are associated with high grade tumours, aneuploid tumours and tumours with a high S phase fraction and high mitotic rate (Isola et al 1992, Allred et al 1993a, Lipponen et al 1993b). Furthermore p53 aberrations are associated with the low levels of oestrogen and progesterone receptors (Cattoretti et al 1988, Mazars et al 1992, Allred et al 1993a, Tsuda et al 1993). As with bladder cancer therefore p53 mutations in breast cancer appear to be associated with factors regarded as demonstrating aggressive disease (Elledge and Allred 1994).

Given this association it is reasonable as with bladder cancer to ask whether p53 has a role as a prognostic or even predictive factor. Until recently the issue has been complicated by a plethora of inconsistent studies using a variety of techniques in varying cohorts of patients (Elledge 1996). Whilst the overall impression especially from larger studies was that p53 mutations did appear to be an independent adverse prognostic factor especially in patients with node negative disease, this was not a universal finding (Elledge et al 1993, Elledge 1996). However a seminal series of papers reporting the prognostic significance of p53 mutations as detected by sequencing of the whole coding region of the gene have enforced the view that p53 mutations do have an adverse

prognostic effect (Bergh et al 1995, Jansson et al 1995, Sjogren et al 1996). Furthermore the work demonstrated that patients with p53 mutations and positive lymph nodes appeared to benefit less from adjuvant therapy especially with tamoxifen (Bergh et al 1995). The demonstration that mutations of p53 at the critical sites for sequence specific DNA binding are associated with primary resistance to adriamycin therapy emphasises that p53 status may have a predictive role in the outcome to specific therapies and that therefore knowledge of p53 status may potentially be used to tailor the choice of therapy for an individual patient (Aas et al 1996).

In summary therefore mutations of p53 are a common event in breast cancer and are appropriate events to study as a potential biochemical marker for the presence of malignancy. As in the studies undertaken on bladder cancer it was decided to confine the screening of mutations to exons 5-8 of the p53 gene as these contain the large majority of mutations described.

1.7 Urine Samples and Bladder Washings In Relation To Bladder Cancer

The epithelium of the bladder, the site of origin of most bladder tumours, is in contact with the urine and epithelial cells are shed into it (Cotran et al 1989a). Whilst cystoscopy with biopsy is the mainstay for the diagnosis and monitoring of patients with bladder cancer cytological evaluation of voided urine samples has an important adjunctive role in some clinical settings (Scher et al 1997). The majority of patients with carcinoma in situ (CIS) of the bladder will exhibit malignant cells in their urine and cytology can be abnormal prior to the appearance of visible endoscopic

abnormalities (Cowan et al 1987, Murphy 1990). Urine cytology is less reliable for those patients with papillary-superficial and invasive bladder cancers due to the small number of malignant cells shed and the heterogeneity of cells present, with cytology being more sensitive for high grade bladder cancers than for low grade cancers (Esposti et al 1978, Cowan et al 1987, Maier et al 1995). Urine cytology samples are particularly difficult to interpret following the use of radiotherapy or chemotherapy (either systemic or intravesical) due to the resultant presence of treatment related cellular dysplasia (Borgmann et al 1993, Wiener et al 1993).

The procedure of bladder washing (in which a small volume of saline is repeatedly injected into and withdrawn from the bladder via a catheter or cystoscope) increases the cellularity of specimens presumably due to the disruption of cells from the urothelial surface by the trauma of the fluid's injection (Walther 1992). The cytological evaluation of bladder washing samples is more sensitive than that of voided urine though there is controversy as to whether or not patients should be catheterised purely to collect such a sample (Murphy 1990, Matzkin et al 1992).

A number of strategies have been adopted to enhance the diagnostic potential of urine samples and bladder washing samples. An early avenue was the use of flow cytometry to assess the DNA content of such samples, the presence of an aneuploid cell population inferring malignancy (Rosai 1993). Whilst it has been suggested that the combined use of cytology and flow cytometry may allow a decrease in the intensity of cystoscopic follow up after treatment (Murphy et al 1986) this has not become standard practice. It has been demonstrated that voided urine samples are probably not suitable for routine clinical use, with bladder

washings providing superior samples (Mellon et al 1991). In a review of the role of flow cytometry in bladder cancer Walther (1992) concludes that the technique may have a role in monitoring response to therapy in patients with CIS, but that it remains suboptimal awaiting further technological and methodological improvements to maximise its utility.

Attempts have been made to enhance cytological evaluation of both urine and bladder washing samples by the use of immunocytochemical staining against a variety of tumour antigens with indications that this may aid in the detection of low grade tumours (Lin et al 1988, Sagerman et al 1994, Tanaka et al 1994). Other approaches have included the measurement of tumour related proteins such as nuclear matrix protein 22 (Miyanaga et al 1997) E- cadherin (Ross et al 1996), cytokeratin fragments (Senga et al 1996) and tumour associated hyaluronic acid (Lokeshwar et al 1997), and the measurement of tumour related enzymes such as beta-glucuronidase (Ho and Kuo 1995) and hyaluronidase (Pham et al 1997), but problems with either sensitivity, specificity or both predominate.

The Bard bladder tumour antigen (BTA) test is a latex agglutination test detecting basement membrane complexes in urine samples that has been the subject of several studies in the diagnosis and assessment of bladder cancer. From these studies it can be concluded that whilst the test is more sensitive than either urine or bladder wash cytology it is less specific in the detection of malignant cells and as such its role, if any, in clinical practice is yet to be established (Ianari et al 1997, Kirollos et al 1997, Sarosdy 1997)).

A promising preliminary report involves the measurement of the activity of telomerase, an enzyme expressed by most cancer cells which is involved in maintaining telomere length and thus enhancing the malignant cells capacity for continuing proliferation. Kinoshita et al (1997) demonstrated telomerase activity in 41 of 42 (98%) bladder cancer specimens and in 55% and 84% of corresponding urine and bladder washing samples respectively, the measurement of telomerase showing better sensitivity than conventional cytology.

Malignancy associated chromosomal aberrations in the urine and bladder washing samples of patients with bladder cancer can be detected by fluorescence in situ hybridisation (FISH) (Meloni et al 1993, Wheelless et al 1994). Though the technique may find a role in diagnosis and follow up of patients with bladder cancer, it's relative lack of sensitivity suggests its major use may lie in the study of the biology of the disease (Wheelless et al 1994).

It is becoming increasingly apparent that the most promising way of maximising the diagnostic use of urine and bladder washings is in the study of molecular markers, and as such it is relevant to review the findings of other workers. A number of approaches have been adopted, all with the common aim of trying to develop and evaluate potential methods using samples gathered by non-invasive or minimally invasive means for the diagnosis and follow up of bladder cancer.

Some bladder cancers contain multiple copies of certain genes such as c-erb B2 and EGF-R and such gene amplification has been demonstrated in a proportion of bladder washing samples from patients with high grade

but not low grade bladder tumours (Lonn et al 1993). Though these results are of interest in establishing the principle that cancer associated genetic changes are demonstrable in such samples, it is noteworthy that all positive samples in this study were abnormal cytologically, and as such the method may have little advantage over conventional pathological studies. Furthermore to be able, by PCR methods, to establish with confidence increased gene copy number requires a high proportion of cancer cells in the sample, a situation that may only occasionally be found.

Using a similar approach Matsumara et al (1994) have described the detection of variant exons of the CD44 gene (an abnormality associated with many tumour types) in a high proportion of urine samples from patients with bladder cancer. The biology of this gene in malignant disease requires further study and whilst the use of this marker should be further explored, concern remains about specificity of the marker in view of the presence of false positive results in 17% of non tumour bearing controls (Matsumara et al 1994). The reliance of the assay on analysing RNA adds to the complexity as this requires meticulous storage and handling of samples to prevent degradation.

Of greater interest is the potential of microsatellite marker analysis in urine samples. Microsatellites are highly polymorphic tandem repeats of short DNA sequences occurring very commonly throughout the genome easily amenable to study by PCR. Using a panel of tri- and tetranucleotide repeats an alteration in at least one of the loci studied in 26% of tumour samples from patients with either head and neck, lung or bladder cancer, has been demonstrated (Mao et al 1994). Furthermore the authors were

able to detect the same alterations in corresponding sputum and urine samples, with dilution assays suggesting that the assay may be able to detect 1 tumour cell in 200-1000 normal cells. Using an expanded panel of markers the same group (Mao et al 1996) demonstrated microsatellite abnormalities in 19 out of 20 patients with bladder tumours, as well as in 2 patients with only atypia on biopsy. The changes found were generally present in both the urine and the tumour. But as many of the markers studied are involved early in the evolution of bladder tumours, and peripheral blood rather than normal bladder mucosa was used as the source for control DNA, it remains possible that the changes found are associated with preneoplastic urothelium rather than being specific to bladder cancer per se. However in a study including some of this cohort it is apparent that the absence of microsatellite abnormalities previously present following treatment is a good predictor of disease free status; furthermore the presence of alterations correlates well with or predates recurrence (Steiner et al 1997). These results merit prospective evaluation with appropriate controls, and whilst employing a large panel of markers the authors feel that the ongoing development of a reliable non-radioactive method would allow rapid introduction of such assays into clinical practice if its utility is confirmed (Steiner et al 1997).

Of relevance to the present study is work performed looking at the possibility of detecting specific tumour associated gene point mutations in urine or bladder washing samples. H-ras is a member of the ras family of oncogenes, and point mutations in this gene are implicated in the biology of bladder cancer, indeed the first documented H-ras mutation was in a bladder cancer cell line (Reddy et al 1982). The frequency of such mutations in bladder cancer is however a contentious issue: initial

reports suggested an incidence of 10-16%, and whilst other series have reported frequencies ranging between 30-75% (Levesque et al 1993, Linehan et al 1997) a large study of 152 cases found an incidence of only 6% (Knowles and Williamson 1993). Additional concerns about the use of H-ras as a disease marker include the observation that mutation may be restricted to only some areas of a proportion of tumours and uncertainty about the significance of H-ras mutations in the biology of bladder cancer (Levesque et al 1993). Despite the above uncertainties a number of interesting studies on H-ras mutations in shed urothelial cells have been reported. Firstly Levesque et al (1993) demonstrated H-ras mutations by SSCP in 4 out of 9 urine or bladder washing samples from patients with bladder cancer. In 1 case tumour, urine and bladder wash DNA were available and all 3 demonstrated the same abnormality, but unfortunately no other corresponding tumour material was analysed. Secondly Fitzgerald et al (1995) identified H-ras mutations in 44% of 100 urine sediments from bladder cancer patients, again using SSCP, but reported data on the mutational status of the corresponding tumour in only 9 cases, 7 of which demonstrated the same mutation. Given the authors observation that, in follow up urine samples, a proportion of patients exhibited a mutation in the absence of visible tumour it is possible that at least on occasions the mutation detected represents a preneoplastic event. A third study using a PCR restriction enzyme based assay reported an incidence of H-ras mutations of 15.2 % of 33 bladder cancers and confirmed the presence of the same mutations in bladder washout specimens (Hong et al 1996). Even if H-ras mutations have limitations as a marker, these studies demonstrate that oncogene mutations can be demonstrated in urine and bladder wash samples.

The potential for using p53 abnormalities as a molecular marker has received some attention. Sidransky and colleagues (1991) demonstrated the same p53 mutations which had previously been characterised in the primary tumour to be present in urine samples from 3 patients with bladder cancer. They used cloning techniques with mutant specific oligonucleotides that by definition required prior knowledge of the specific mutation being sought in the urine. The theoretical usefulness of such mutation detection was illustrated by the retrospective demonstration of the same p53 mutation found in the cystectomy specimen of a former United States Vice President, Hubert Humphries in a cytologically negative urine sample taken 9 years earlier (Hruban et al 1994). However with the widespread nature of the potential sites for p53 mutations within the gene, p53 mutant specific oligonucleotide probes are not a viable screening tool for the early diagnosis of bladder cancer but may have a role in follow up of patients whose primary tumour contains a characterised mutation. Two recent papers, both published after the current study was underway, illustrate the potential for studying p53 in bladder washings or urine samples. In the first p53 abnormalities were demonstrated in the bladder washings of 6/13 patients with superficial bladder cancer who progressed to invasive disease but only in 1/13 who did not progress. SSCP was used as the mutation screening method and the authors were able to demonstrate the presence of p53 mutations in washings several months prior to the development of invasive disease, and therefore concluded that p53 mutations may be important in the transition from superficial to invasive disease. The p53 status of the primary tumour was assessed in only 2 cases. In both of these a p53 mutation had been demonstrated in bladder washings prior to the development of invasive disease. The same mutations were found in the

subsequent invasive tumours, but interestingly not the bladder washing samples taken when invasive disease was found. Whilst these data are preliminary they confirm, as does the current study, the feasibility of detecting and characterising p53 mutations in bladder washing samples (Vet et al 1996).

The second study identified p53 mutations in tumour samples from 13/28 patients with multifocal bladder cancer. Thirty urine samples from 8 patients with mutations were collected at diagnosis and during 2 years of follow-up. Twenty four of these demonstrated the same mutation as in the primary tumour; the 6 negative samples coincided with negative cystoscopies, as interestingly did 6 of the positive urine samples. Of these latter patients 2 were lost to follow up and the other 4 developed recurrence within a few months suggesting the maintenance of an abnormal exfoliating tumour cell population in the absence of obvious tumour. In this study the mutation detection was all performed by direct sequencing, a method possibly less sensitive than SSCP (Smith et al 1992), and it was estimated that the mutation carrying cells represented between 20 % and 70% of the sample (Xu et al 1996) in contrast to the 1-7% estimated by Sidransky et al (1991).

A further important study on bladder washings assessed p53 expression immunocytochemically using flow cytometry. Abnormal p53 expression was found in 24% of 90 bladder washing samples from patients with superficial bladder tumours and 33% of 12 samples from patients with invasive disease (Griffiths et al 1995). The concordance between p53 expression in washings and corresponding tumours was only 74%, and the authors suggest that dual parameter analysis with a second marker

such as cytokeratin may improve sensitivity by enabling only epithelial cells to be analysed. It must be emphasised that the primary objective of this study was to assess p53 expression in bladder washings as a prognostic marker rather than as a marker of the presence or absence of disease; however it was found that abnormal DNA content was of greater predictive value (Griffiths et al 1995). The final reported study on p53 and urine samples has adopted a different approach. Short term culture of urinary exfoliated cells was performed on samples from 52 patients with urothelial tumours. Successful passage was obtained in 77% of cases, there was over 90% concordance between immunohistochemical analysis of the tumour and the urinary cultures and it is suggested as a non-invasive method for early detection and follow up of bladder tumours (Okuno et al 1996). However such a method, even if high specificity and sensitivity could be demonstrated, is unlikely to find a clinical role in view of the technical demands of such a strategy.

Given the above, further studies on the potential use of the detection of specific gene mutations as a marker for the presence of malignancy are justified in patients with bladder cancer. Bladder washings were used in this study because of the improved cellularity discussed above, maximising the chances of detecting mutations in shed epithelial cells. This was felt particularly important for a pilot study given the low proportion of mutant carrying cells described in urine samples by Sidransky et al (1991). The reasons for choosing p53 mutations as a marker are discussed earlier.

1.8 Breast Ductal Secretions and Nipple Aspirate Fluid

The developed female breast secretes and absorbs fluid constantly even in the resting state (Petrakis 1986). This fluid is secreted by the alveolar ductal system of the breast and is absorbed by the same system, the fluid therefore being in intimate contact with the epithelium that gives rise to the majority of breast cancers (Wynder and Hill 1977). Perhaps surprisingly this fluid and the epithelial cells shed into it have historically received little attention from clinicians and scientists interested in breast cancer, the fluid being regarded as an uninteresting proteinaceous material containing sloughed off degenerative cells of little importance (Petrakis 1986). However over the last 20 years interest has increased, largely due to the development of a simple and reliable method for sampling this fluid (Sartorius et al 1977). The method is described in detail in Chapter 2 (section 2.3.2), but briefly involves the application of negative pressure to the cleaned nipple by means of a plastic cup attached to a syringe. The resulting fluid is collected from the surface of the nipple in a suitable tube for storage (Sartorius et al 1977). The method has been widely used in an outpatient setting and is safe, reliable and well tolerated by patients (Sartorius et al 1977, King et al 1983, Dairkee and Hackett 1986), and yields a fluid sample in a much greater proportion of patients than a previous method using a maternity breast pump (Papanicolaou et al 1958).

Since the introduction of the Sartorius method a substantial body of data has been reported regarding breast fluid obtained by nipple aspiration. The fluid is referred to as Nipple Aspirate Fluid (NAF) to distinguish it from the fluid obtained from aspiration of breast cysts. Before reviewing

some of this data and its relationship to breast cancer, it is important to review the reliability of the technique in obtaining a sample of fluid and the characteristics of the subject that influence this.

Whether a sample is useful for clinical or research purposes depends on the proposed use of the fluid. In broad terms studies on NAF can be divided into those investigating the cellular characteristics of the fluid using cytological techniques and those investigating its biochemical properties. The former requires a sample containing well preserved cells whilst meaningful biochemical studies can be performed on small acellular samples (Gruenke et al 1987a). In an early study involving 1503 women fluid containing sufficient cells for cytological evaluation was obtained from 55% of subjects with a further 11% yielding fluid unsuitable for cytological study due the scarcity of ductal epithelial cells or the degenerate nature of those cells present. 65% of women aged 31-50 years yielded cellular fluid samples (Sartorius et al 1977). Furthermore in a separate study fluid was obtained from 76% of Caucasian women under 51 years of age and 55% of Caucasian women 51 years and older (Petrakis et al 1975). A review pooling results from 6 studies describes four features that are consistently associated with the successful obtainment of NAF: 1) age 35-50 years, 2) early age of menarche, 3) non- asian ethnic origin and 4) previous lactation. The studies revealed that the effect of parity could be accounted for by breastfeeding and the effect of age was independent of menopausal status (Wrensch et al 1990). It is therefore reasonable to conclude that a cohort of suitably aged subjects will yield a sample of fluid in the majority of cases. It may also be of relevance that the fluid obtained from women with breast disease tends to be more cellular than that from normal breasts (King et al 1975).

As stated above studies of NAF can be divided into those concentrating on either the cellular or biochemical aspects of the fluid; both will be considered in turn, along with relevant data from studies on spontaneous discharging breast fluid. Breast fluid from patients with symptomatic breast discharge can be of diagnostic value and there are numerous historical reports the primary diagnosis of breast cancer being made on the basis of cytological smears of such fluid (Papanicolaou et al 1958) . In a recently published series auditing the use of nipple discharge cytology 7/15 breast cancers from 338 patients with nipple discharge were correctly identified by cytology and it was concluded that whilst examination of such fluid was a useful adjunct to other diagnostic modalities it was not safe to rely on it as the sole modality of investigation of nipple discharge (Groves et al 1996). It is noteworthy also that the majority of patients with spontaneous nipple discharge do not have breast cancer. The main cellular components of breast ductal fluid (obtained either by nipple aspiration or spontaneous discharge) are ductal epithelial cells, foam cells, macrophages, blood cells (most commonly white cells) and squamous epithelial cells (Papanicolaou et al 1958). Foam cells are the most frequently occurring cell type and whilst their histiogenic origin remains unclear the available evidence points towards a phagocytic rather than epithelial derivation. (Petrakis 1986). Foam cells are regarded as having little diagnostic value, however it is of interest that in one study a model based on cytometric image analysis of foam cells correctly placed 83% of patients with a variety of malignant and benign breast pathologies in the correct diagnostic group whilst convention cytological criteria failed to demonstrate any distinguishing features (King et al 1984).

In terms of cytology most interest has centred on the ductal epithelial cells

present in NAF. In an early series reporting on the cytology of NAF 27 fluids were classed as suspicious of carcinoma, and the diagnosis of cancer was confirmed in 18 (66%) of these patients. Of these 18, 7 were diagnosed by aspirate cytology alone the patients all having normal mammography and clinical examination (Sartorius et al 1977). Subsequently in a further study of 1744 asymptomatic subjects 280 satisfactory samples revealed 8 with atypical cells with only 1 subject being confirmed as having a carcinoma (Buehring 1979). Using strict cytological criteria for the diagnosis of malignancy 21% of 34 patients with a known malignancy could have their diagnosis confirmed by aspirate cytology (King et al 1983). Perhaps these authors more important finding was the strong association of cells showing features of atypical hyperplasia in NAF samples with atypical ductal hyperplasia in the patients' corresponding operative breast tissue specimen, both in the absence and presence of coexisting malignancy. In view of the significant association between atypical ductal hyperplasia and the subsequent development of breast cancer (Page et al 1985) these abnormal hyperplastic cells in aspirate fluid could reasonably be hypothesised to be a marker of breast cancer risk. Furthermore it was noted that the fluid from 70% of the patients with a malignancy demonstrated such cells (King et al 1983).

It has been demonstrated that there is an association between the presence of dysplastic epithelial cells in NAF and the subject having a first degree relative with breast cancer. This association is strongest when other breast cancer risk factors, such as gross fibrocystic disease are also present, implying either an additive or synergistic adverse effect of these factors and family history on the breast epithelium (Petrakis et al 1982). Subsequently the same group demonstrated a significantly increased

incidence of breast cancers developing during follow up of a cohort of 420 women with cells displaying atypical hyperplasia in nipple aspirate fluid as compared to an age and race matched group of controls with "normal" fluid (Petrakis et al 1987a). A further prospective cohort study, with an average of 12.7 years of follow-up, on 2700 white women without symptoms of breast disease demonstrated an increased risk of breast cancer development in certain patient groups (Wrensch et al 1992). The authors assessed any fluid obtained cytologically and correlated the findings with subsequent development of breast cancer. The subjects were divided into 5 groups as follows: no fluid obtained, fluid cytologically unsatisfactory, fluid with normal cytology, fluid showing hyperplasia and fluid showing atypical hyperplasia. The risk of breast cancer developing during the study was lowest in those from whom no fluid was obtained. The relative risk was significantly higher in those with epithelial hyperplasia (2.5 , 95% confidence interval 1.1 - 5.5) and those with atypical hyperplasia (4.9, 95% confidence interval 1.7 - 13.9). Additionally those with a first degree relative with breast cancer and atypical hyperplasia appeared to be those most at risk (Wrensch et al 1992). Nodular densities on mammograms have been described as a marker of increased breast cancer risk (Wolfe et al 1983) and a relationship between high density mammograms and cytological atypia in NAF specimens has been reported (Lee et al 1994).

Collectively these data strongly support the hypothesis that the presence of dysplastic cells in nipple aspirate fluids is associated with an increased risk of breast cancer. Whilst it can be concluded that the cytological study of nipple aspirate fluids is not a suitable modality to be used as primary diagnostic tool, malignant cells can be detected in a proportion of women

with breast cancer and cells associated with subsequent breast cancer risk are readily identifiable. Unfortunately to date the specificity and sensitivity are not of the required level to guide intervention at an individual patient level if the cytological examination of NAF were to be considered as either a screening tool or primary diagnostic tool. However the cellular material present in NAF may well serve as a suitable source of DNA and/or RNA for use in molecular biological studies.

Nipple aspirate fluid is biochemically complex containing a variety of chemical species including proteins, lipids, carbohydrates, both steroid and peptide hormone and a plethora of exogenous substances (Rose 1986, Petrakis 1993). Studies on the biochemistry of NAF have given some useful insights into the function of the breast and how function may relate to disease. Sanchez et al (1992) characterised NAF by gel electrophoresis and protein sequencing into 2 classes, types I and II. Whilst all fluids may contain albumin, type I fluids are closely related in protein composition to cyst fluid (containing Zn α_2 glycoprotein, apolipoprotein D and gross cystic fluid protein 15) and type II fluids are similar to milk (containing lactoferrin, lysozyme and lactalbumin). Furthermore type I fluids were present in 93% and 88% of patients with no breast disease or benign breast disease respectively with only 43% of patients with malignant breast disease having a type I fluid. These different patterns possibly reflect differences in the hormonal environment of the breast in benign or malignant settings (Sanchez et al 1992). Breast fluid contains immunoglobulins including Ig G, Ig M and Ig A, the latter being in the secretory form and often at higher levels than in plasma (Petrakis 1977, Yap et al 1981).

Given the hormonal influences on the breast in health and disease, the hormonal composition of nipple aspirate fluid is of considerable interest. Oestrogen, prolactin (Wynder and Hill 1977), progesterone (Rose et al 1986), testosterone (Hill et al 1983) and dihydroepiandrosterone sulfate (Miller et al 1981) are detectable often at higher levels than in plasma, and it would appear that ductal epithelial cells can actively transport steroid and peptide hormones against a concentration gradient (Wynder et al 1981). Breast fluid oestrogen concentrations are many fold higher than those in serum and whilst serum levels fall with the menopause NAF levels remain high (Ernster et al 1987, Petrakis et al 1987b). Additionally NAF oestrogen levels are lower in premenopausal parous women with levels increasing gradually over several years after the cessation of breastfeeding (Petrakis et al 1987b). Therefore the reduced risk of breast cancer in those who have breast fed may in part be related to lower cumulative exposure to high oestrogen levels (Ernster et al 1987, Petrakis et al 1987b). Increased concentration of testosterone in breast fluid has been reported in breast cancer patients compared to healthy controls (Hill et al 1983).

It is clear therefore that breast fluid has it's own hormonal microenvironment. This may well be relevant to disease processes within the breast because of the potential effect of the hormones on the ductal epithelium. The above data illustrate that serum levels of hormones give only at best a crude picture of the hormonal environment of the breast and that a different and potentially more relevant picture can be obtained by the study of NAF.

A major lipid component of breast fluid is cholesterol and significant

quantities of cholesterol and cholesterol's oxidative breakdown products can be detected (Petrakis et al 1981). There is evidence from model systems that cholesterol epoxides (the main group of breakdown products) are both mutagenic and carcinogenic (Morin et al 1991, Petrakis 1993). It 's therefore of interest that there appears to be an association between increased levels cholesterol epoxides and proliferative benign breast disease (Wrensch et al 1989). Furthermore concentrations of both cholesterol and cholesterol beta epoxide are reduced in breast milk with this reduction being detectable in NAF for up to 2 years following the cessation of breast feeding (Gruenke et al 1987b). It has therefore been postulated that the alveolar ductal system of parous women who have breast fed are exposed to lower cumulative levels of such potential toxins and that this may be relevant to the lower incidence of breast cancer in women who have breast fed (Gruenke et al 1987b). In this context it is of interest that the Tanka women of Hong Kong who suckle their infants only from the right breast have an increased incidence of left sided breast cancers suggesting that stagnation of toxins may be important etiologically (Ing et al 1977). Certainly there is additional evidence that toxins are accumulated by the breast, for example nicotine and cotinine (a nicotine metabolite) are detectable in breast fluid within half an hour of smoking (Petrakis et al 1978, Hill and Wynder 1979). Additionally a proportion breast fluid samples from both non pregnant and pregnant women are Ames test positive indicating the presence of mutagenic agents in the fluid (Petrakis et al 1980, Petrakis 1986, Scott and Miller 1990). As is the case with hormones these data imply that the local environment of the ductal epithelium is directly affected by the contents of breast fluid.

The possibility that cancer associated antigens may be found in nipple

aspirate fluid and correlate with disease status has been explored. Prostatic specific antigen (PSA) was previously thought to be exclusively found in the prostate but is now known to be present in some breast tumours (Diamandis et al 1994) and to be associated with a good prognosis (Yu et al 1995). Recently PSA has been detected in NAF samples (Sauter et al 1996). A cohort of women with invasive breast cancer or at variable risk of developing breast cancer were studied. The levels measured varied widely but an inverse relationship was found between PSA levels and breast cancer risk (Sauter et al 1996). The levels of carcino embryonic antigen (CEA) have been measured in the breast fluid of 26 women with spontaneous breast discharge. All 4 patients subsequently diagnosed as having an underlying breast cancer were members of a group of 7 cases exhibiting the highest CEA levels (Kahana et al 1981). The authors concluded that breast fluid CEA measurement may have an adjunctive role in the diagnosis of patients with symptomatic nipple discharge, though in this context CEA was showing features of a tumour-associated rather than tumour-specific protein.

Epidermal growth factor (EGF) is a mitogen for mammary cells and transforming growth factor alpha is a related polypeptide involved in the autocrine regulation of breast cancer (Dickson and Lippman 1997). Both have been detected in human milk and in nipple aspirate fluid from nonlactating women. The preliminary data suggest that EGF levels are higher in subjects with benign breast disease compared to normal controls (Rose et al 1992) and whilst there is currently no data indicating whether or not levels of these factors are related to the risk or presence of breast cancer further study is warranted (Vogel et al 1992)

Attention is turned finally to the molecular biology of breast fluid. Given the nature of the fluid and its association with breast epithelium it is reasonable to hypothesise that useful information may be gained by the use of molecular biological techniques to study nipple aspirate fluid. This potential has however not been widely explored. A Japanese group have reported increased levels of the c-erb B2 oncoprotein (a member of the epidermal growth factor receptor family) in the breast fluid of patients with symptomatic spontaneous breast discharge and breast cancer as compared to benign disease, and also found a good correlation between the protein levels in the tumour and fluid (Inaji et al 1993). Furthermore the same group were able to demonstrate by PCR the amplification of the c-erb B2 gene in discharge fluid of a patient with a non palpable breast carcinoma, a similar level of amplification being found in the tumour but not in white blood cells (Motomura et al 1994).

To date only 1 study has been reported investigating p53 in breast fluid. Using an immunohistochemical method no staining for p53 was found in any 19 smears of breast fluid obtained from women with breast discharge, but staining indicative of abnormal or mutant p53 was found in 19/40 fine needle biopsy specimens from patients with breast cancer (Koutselini et al 1991). The report does not clearly state the diagnoses of the patients with nipple discharge but by implication they would all appear to have had benign disease. There have been no published reports on p53 and breast fluid obtained by nipple aspiration.

It can therefore be seen that although there are a few small studies investigating oncoproteins and growth factors there have been no data describing the detection of mutations in specific oncogenes or tumour

suppressor genes in nipple aspirate fluids (or spontaneous breast discharge) published.

Preliminary work using specific primers have allowed the amplification by the polymerase chain reaction (PCR) of DNA from the genes for aromatase, a interferon c-erb B2 and p53 (P Sourdain, personal communication of unpublished data) and one of the major aims of this project was to investigate whether mutations of the p53 gene were detectable in nipple aspirate fluid from patients with breast cancer.

1.9 Mutation Detection and Single Stranded Conformational Polymorphism (SSCP)

The studies on clinical samples described in subsequent chapters centred on the detection of mutations in the p53 gene. As has been discussed, the majority of p53 mutations described to date have been single base point mutations, rather than large deletions or other gross aberrations (Harris and Hollstein 1993). Furthermore the mutations found, whilst concentrated in the more highly conserved regions of the gene are still present over a large proportion of the coding sequence (Soussi et al 1990, Hollstein et al 1991). These factors are thus of great importance in the choice of an appropriate mutation detection methodology. Direct sequencing of PCR amplified fragments of DNA is regarded as the "gold standard" method of mutation detection being sensitive and definitive with the presence of a mutation being identified and characterised in the same process (Sklar and Costa 1997). The major disadvantage is the technical complexity of producing large runs of readable sequence combined with the time consuming and resource intensive nature of the

technique when used on a large number of samples (Sklar and Costa 1997). A number of methods have been developed to screen for the presence of unknown mutations, allowing the identification of mutation carrying samples, with the mutation subsequently being characterised by direct sequencing, and include (Grompe 1993, Prosser 1993, Sklar and Costa 1997):

- 1) Single Stranded Conformational Polymorphism analysis (SSCP)
- 2) Denaturing Gradient Gel Electrophoresis (DGGE)
- 3) Chemical Mismatch Cleavage (CMC) and a variant of this using Carbodiimide (CDI)
- 4) Heteroduplex analysis
- 5) RNase A cleavage

None of these screening methods is ideal and the choice of method employed in any particular study will depend on the nature of the samples being studied, the size of the products of interest, the degree of sensitivity required as well as the resources and technical expertise available (Grompe 1993). With the likely increasing availability of automated direct sequencing, the role of such screening techniques may in many situations become redundant at some stage in the future, (Grompe 1993, Prosser 1993). Currently however screening tests continue to be an efficient way of maximising rapid mutation detection in terms of both resources and labour-intensity.

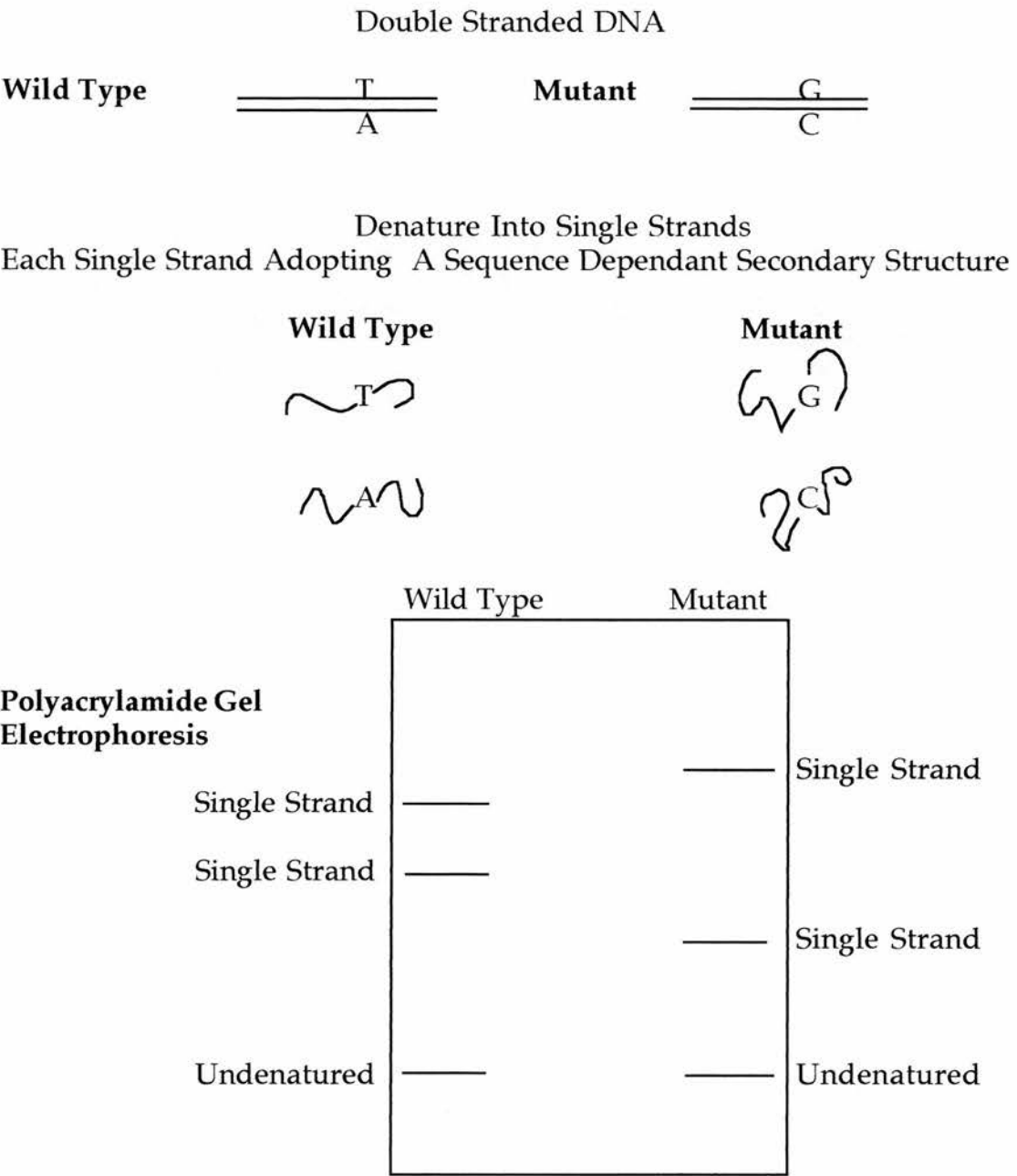
It is not appropriate to review in detail the underlying principles of all the above techniques, but the major advantages and disadvantages will be briefly summarised as they are relevant to the choice of method that was made.

The most obvious limitations of RNase A cleavage are the requirement for radiolabelled RNA and the techniques limited sensitivity being able to detect only 50-60% of mutations (Grompe 1993, Prosser 1993). CDI and CMC are sensitive techniques but labour intensive and utilise hazardous chemicals and as such have not found widespread use (Grompe 1993, Prosser 1993). Heteroduplex analysis whilst easy to perform probably is less sensitive than optimised SSCP in mutation detection (Prosser 1993). DGGE relies on the detection of the differences in migration as PCR products gradually denature as they pass through a gel containing an increasing concentration of denaturing agents such as urea and formamide (Fischer and Lerman 1983). The sensitivity of the technique has been greatly enhanced by the attachment a 40 base pair GC rich sequence (GC clamp) to the upstream end of one of the PCR primer pair preventing the clamped end denaturing and thus accentuating migration differences (Sheffield et al 1989). The technique requires the computer assisted generation of primer sequences that ensure an appropriate melting profile for the amplified product so that the likelihood of mutation detection is maximised. Primers have been designed to cover the entire coding regions of exons 5-8 of the p53 gene and it has been suggested that the sensitivity of this technique approaches 100% (Moyret et al 1994). However use of the widely used primers designed by Borresen and colleagues (Borresen et al 1991) which cover the conserved regions but not the entirety of exons 5-8 would have failed to detect several of the mutations found in the current study. The major disadvantage of the technique is the effort required to establish the method for the particular PCR products of interest (Prosser 1993). One potential advantage of the technique in general terms was seen as a potential disadvantage for the present study, namely the products are visualised by non isotopic

methods which may have a limiting effect on the ability to detect rare mutation carrying products when DNA from a mixture of wild type and mutation carrying cells is studied.

SSCP was first described in 1989 (Orita et al 1989 a, b) and has subsequently become the most widely used mutation screening technique (Grompe 1993). It relies on the separation of a double stranded DNA product into 2 radiolabelled single strands prior to electrophoresis on a non denaturing polyacrylamide gel. Under such conditions the single strands form their own sequence dependant secondary structure which determines the mobility of the fragments on electrophoresis, with point mutations potentially leading to mobility shifts relative to wild type strands. In addition some of the DNA reanneals to reform double stranded DNA whose migration on the gel is determined by size rather than sequence. The principle of SSCP is shown schematically in Figure 1.3 (page 64). Under appropriate conditions SSCP may detect from 75-98% of mutations (Ravnik-Glavac et al 1994). Sensitivity of SSCP is improved when gels with a high concentration of acrylamide and low concentration of crosslinker (bisacrylamide) are employed (Glavac and Dean 1993) and mutation detection is enhanced by the use of more than one set of gel conditions, including the addition of glycerol to the gel and the running of gels at different temperatures (Orita et al 1989 a,b, Glavac and Dean 1993, Vidal-Puig et al 1994). The size of PCR product studied has a profound effect on sensitivity (Sheffield et al 1993) and it has been estimated that for products of around 200 base pairs or less sensitivity is approximately 90% falling to 80% for 300-350 base pair products (Hayashi and Yandell 1993). It is accepted that the optimisation of SSCP is largely

Figure 1.3
Schematic Depiction of the Principle of SSCP



(Adadpted from Prosser 1993)

empirical and has to be established for each primer pair (Glavac and Dean 1993, Prosser 1993), with the best results obtained when 2 or 3 sets of gel conditions are used (Hayashi and Yandell 1993, Vidal-Puig et al 1994). The wide body of literature supporting its use, its applicability to PCR products of the length of interest in p53 mutation detection, the comparative simplicity of the technique and the use of product radiolabelling (allowing the potential to detect low intensity electrophoretic bands by prolonged autoradiography in samples containing a low proportion of tumour derived DNA) all contributed to the decision to use SSCP as the mutation detection method in the present study. A further attraction was the potential to excise mutation carrying bands from dried gels for the purposes of sequencing, a major benefit when only a proportion of the sample carries the mutation (J Prosser, personal communication).

One further method that has received attention is Dideoxy Fingerprinting (ddF) a hybrid between dideoxy sequencing and SSCP (Sarkar et al 1992). In this technique the product of 1 Sanger dideoxy chain terminating sequencing reaction is subjected to electrophoresis through a non denaturing polyacrylamide gel. This enables the presence of a mutation to be inferred by the loss or gain of a termination segment and /or by the presence of a mobility shift of one or more termination segments due to conformational changes caused by the mutation. This method has a high degree of redundancy, has a sensitivity approaching 100% and has been demonstrated to be efficient in the detection of p53 mutations (Blaszyk et al 1995). The predominant reasons for not using it in the place of SSCP in this study were concern over the potential difficulty in interpreting the gels with the multiplicity of bands that could be envisaged in samples containing a mixture of wild type and mutant product, as well as the

inferior ability demonstrated for direct sequencing to demonstrate the presence of mutations in such a mixture compared to SSCP (Smith et al 1992, Wu et al 1993).

1.10 AIMS OF STUDY

The study set out to investigate a number of issues. Since p53 mutations are amongst the most common genetic aberrations described to date in a wide variety of cancer types including bladder cancer and breast cancer there is potential for studying such genetic changes in exfoliated tumour cells sampled in a non-invasive or minimally-invasive manner to serve as a potential marker of malignancy.

The objectives of the study were thus as follows:

- 1) To develop a PCR-SSCP assay to screen exons 5-8 of the p53 gene for the presence of mutations.
- 2) To optimise conditions for mutation detection and define the sensitivity of the assay to detect the presence of mutation in mixtures of mutation- and non mutation-carrying cells (as the clinical samples to be later studied would almost certainly contain such a mixture).
- 3) To determine whether p53 mutations could be detected in bladder washings obtained from patients with bladder cancer, correlating the findings with those from corresponding tumour DNA.
- 4) To investigate whether p53 mutations could be detected in the nipple aspirate fluid of patients with breast cancer, correlating the findings with those from corresponding tumour DNA.
- 5) To define objectives for future studies.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

The reagents used and their suppliers are listed below alphabetically and are followed by a list of the suppliers' addresses. Where appropriate analytical grade or electrophoretic grade reagents were used.

<u>Reagent</u>	<u>Supplier</u>
Acetic Acid (Glacial)	BDH Merck
Acrylamide 40% solution	Scotlab
Agarose	Gibco BRL
Ammonium Persulphate	Sigma
Bisacrylamide 2% solution	Scotlab
Boric Acid	Sigma
Bromophenol Blue	Sigma
α - ³⁵ S-2'-Deoxy-Adenosine-5'-Triphosphate (³⁵ S-dATP, α S, > 1000Ci/mmol)	Amersham
2'-Deoxy-Adenosine-5'-Triphosphate	Boehringer Mannheim
α - ³² P-2'-Deoxy-Cytidine -5'-Triphosphate Redivue (α - ³² P dCTP)	Amersham
2'-Deoxy-Cytidine -5'-Triphosphate	Boehringer Mannheim
2'-Deoxy-Guanosine-5'-Triphosphate	Boehringer Mannheim
2'-Deoxy-Thymidine-5'-Triphosphate	Boehringer Mannheim
Dimethyldichlorosilane Solution	BDH Merck
Dimethyl Sulphoxide (DMSO)	BDH Merck
Dithiothietol (DTT)	Sigma

DNA Ladder (100 Base Pair)	Gibco BRL
Dulbecco's Modified Eagle's Medium	Gibco BRL
Easigel Sequencing Gel Mix	Scotlab
Ethanol	BDH Merck
Ethidium Bromide	Sigma
Ethylenediaminetetraacetic Acid (EDTA)	Sigma
Foetal Calf Serum	Gibco BRL
Formamide	BDH Merck
Glycerol	Fisons
Isopropanol	BDH Merck
Kodak X-OMAT XAR-5 film	Sigma
Light Mineral Oil	Sigma
Magnesium Chloride (25mM)	Promega
Magnesium Chloride (Salt)	Sigma
Methanol	BDH Merck
Mutation Detection Enhancement (MDE) Gel Solution	FMC BioProducts
Oligonucleotide Primers	I.C.R.F.
PCR Reaction Buffer, 10x (no Magnesium Chloride)	Promega
PIC/Taq DNA Polymerase	I.C.R.F.
Phosphate Buffered Saline Tablets	Unipath
Proteinase K	Sigma
QIAamp tissue kit	Qiagen
QIAquick gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
RPMI 1640 Medium	Gibco BRL
Sequenase Version 2.0 (DNA Sequencing Kit)	Amersham

Sodium Acetate	Sigma
Sodium Dodecyl Sulfate (SDS)	Fisons
Sodium Hydroxide	BDH Merck
N,N,N,N, Tetramethylethylenediamine (Temed)	Sigma
Tris(hydroxymethyl)aminomethane (Tris Base)	Sigma
Tris(hydroxymethyl)aminomethane Hydrochloride (Tris HCl)	Sigma
Xylene	Fisons
Xylene Cyanol FF	Sigma

Addresses of Suppliers

Amersham Pharmacia Biotech UK Ltd, Amersham Place, Little Chalfont,
Bucks, U.K.

BDH Merck, Hunter Boulevard, Magna Park, Lutterworth, Leics, U.K.

Biorad Laboratoris Ltd, Biorad House, Maylands Avenue, Hemel
Hempstead, Herts, U.K.

Boehringer Mannheim, Diagnostics and Biochemicals Ltd, Bell Lane,
Lewis, East Sussex, U.K.

Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, Leics,
U.K.

FMC BioProducts, 191 Thomaston Street, Rockland, Maine, USA.

Gibco BRL Life Technologies, Fountain Drive, Inchinnan Business Park,
Paisley, U.K.

Imperial Cancer Research Fund, Central Services, Oligonucleotide Synthesis Laboratory, Clare Hall Laboratories, Blanche Lane , South Mimms, U.K.

Promega U.K. Ltd., Delta House, Chilworth Research Centre, Southampton, U.K.

Qiagen UK Ltd, Boundary Court, Gatwick Road, Crawley, West Sussex, U.K.

Scotlab Biosciences Ltd, Kirkshaws Road, Coatbridge, Strathclyde, U.K.

Sigma Chemical, Fancy Road, Poole, Dorset, U.K.

Techne (Cambridge) Ltd, Duxford, Cambridge, U.K.

Unipath Ltd, Basingstoke, Hampshire, U.K.

2.2 Buffers and Dyes

The following Buffers and Dye solutions were used:

DNA Loading Dye:

0.25% bromophenol blue

0.25% xylene cyanol ff

30% glycerol in distilled water

Phosphate Buffered Saline:

Prepared by adding 10 Phosphate Buffered Saline tablets to 1 litre of distilled water.

SSCP Loading Dye:

95% formamide

0.1% bromophenol blue

0.1% xylene cyanol ff

5mM NaOH

Tris Acetate Buffer (TAE):

50x stock solution pH 8.0:

242 gm Trizma Base

100 ml, 0.5M EDTA pH 8.0

57.1 ml, Glacial Acetic acid

made up to 1 litre with distilled water

Tris Borate Buffer (TBE):

10x stock solution pH 8.0:

108 gm, Trizma Base

55 gm, Boric Acid

40 ml, 0.5M EDTA pH8.0

made up to 1 litre with distilled water

2.3 Subjects and Sample Collection

2.3.1 Nipple Aspirate Subjects

As described in the introduction (section 1.8) several factors have been reported to be associated with the ability to collect nipple aspirate fluid samples from female subjects, including younger age , non-Asian ethnicity increasing parity and previous breastfeeding. With these in mind the following recruitment criteria were used: all subjects were female patients aged 55 years or younger with a histologically confirmed diagnosis of breast cancer who had not had any previous treatment for their cancer or previous surgery to that breast. These criteria were chosen to maximise the numbers recruited whilst maintaining a reasonable likelihood of any individual subject yielding a fluid sample. All subjects had given written informed consent, having had the nature of the procedure and the purpose of the research explained to them and having read the subject information sheet (see Appendix 1 for consent form and subject information sheet).

2.3.2 Nipple Aspirate Collection

Nipple aspirate samples were collected as follows employing a modification of the technique originally described (Sartorius et al 1977). . The aspiration technique has been widely used without anaesthesia, however as all subjects in the study were scheduled to have an anaesthetic for their planned operation, the samples were collected immediately after the induction of anaesthesia to minimise subject inconvenience. The nipple was cleaned with an alcohol impregnated swab and the perspex suction cup placed over the nipple. The perspex

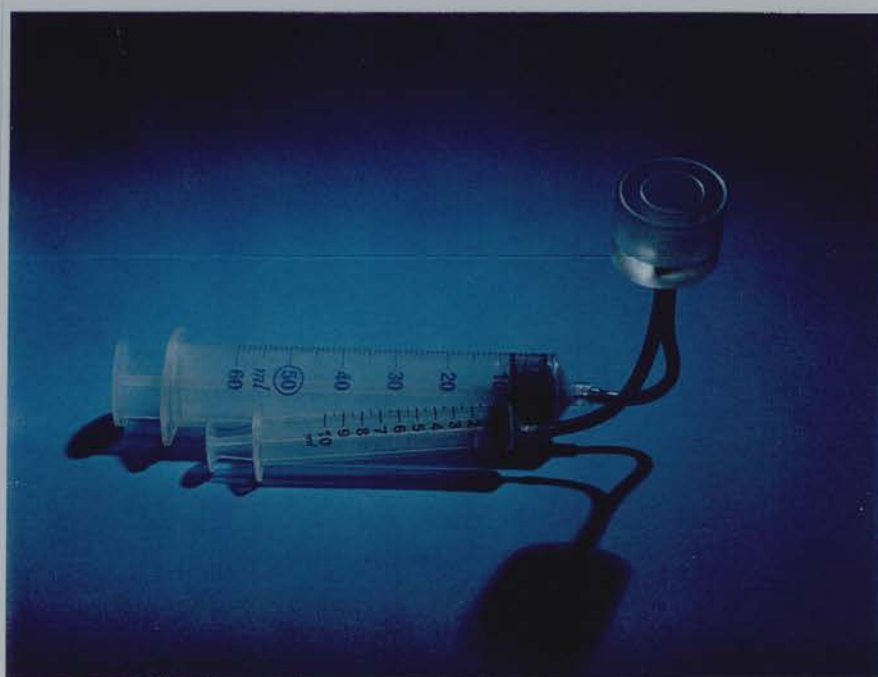
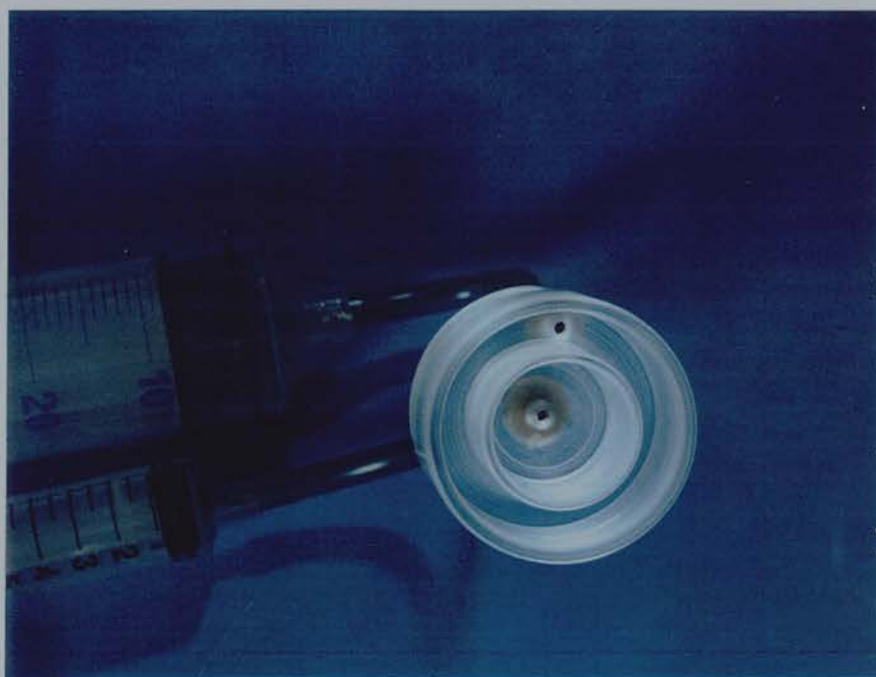
suction cup is shown in Figure 2.1. It consists of an inner chamber 1.7 cm in diameter and an outer concentric chamber 2.9 cm in diameter, each chamber having an adaptor/port allowing a syringe to be attached. The outer port of the suction cup was attached by flexible plastic tubing to a 10ml. syringe and the central port to a 50ml. syringe. Negative pressure was applied via the outer port creating a seal between the skin of the areola and the suction cup. Negative pressure was then created over the nipple by gently and gradually withdrawing the the plunger of the 50ml syringe to approximately the 20 ml mark. This negative pressure was maintained for approximately 20 seconds and then released. Any fluid expressed was then collected in a glass capillary tube and the tube sealed at both ends with plasticene, or alternatively the fluid was aspirated into a 0.65ml microfuge tube using a 200 μ l micropipette and a sterile disposable tip. The tube was labelled and stored at -20⁰C prior to analysis. If no fluid was visible the process was repeated on a further 2 or 3 occasions at which stage if no fluid was obtained the subject was deemed a non-secretor . To avoid contamination of the samples, gloves were worn at all times when collecting and handling the sample. The suction cups were cleaned thoroughly with detergent, rinsed in water and air dried after use and then wiped with an alcohol wipe prior to use.

2.3.3 Breast Tumour Samples

Breast tumour specimens were obtained for DNA extraction from all patients from whom a nipple aspirate fluid sample was obtained. At the completion of the subject's operation the resected specimen was examined fresh by the duty pathologist. The tumour was identified and

Figure 2.1

Photographs of the apparatus used for collection of nipple aspirate fluid samples. The perspex suction cup has inner and outer chambers each with it's own adaptor allowing the attachment of a syringe by flexible plastic tubing.



separate samples dissected for histological evaluation and for oestrogen receptor assessment. If sufficient tumour was available an additional sample was snap frozen in a cryotube in liquid nitrogen for the purposes of the current study and the tissue stored in liquid nitrogen until the time of DNA extraction. In those cases in which there was insufficient tumour DNA was obtained by extraction from paraffin embedded rather than fresh tumour.

2.3.4 Breast Tissue-Normal

In a small number of patients a separate sample of macroscopically normal (i.e non-malignant) breast tissue was also taken and stored in liquid nitrogen until the time of DNA extraction.

2.3.5 Bladder Washouts: Subjects and Sample Collection

Patients were identified from cystoscopy operating lists as having a possible or definite diagnosis of invasive bladder cancer. Such operations were attended by the investigator and bladder washout samples gathered as follows: the subject was cystoscoped under general anaesthetic and if the presence of a bladder tumour was confirmed a bladder washout was performed prior to tumour resection. The bladder was drained via the cystoscope and a 60ml bladder syringe filled with normal saline was attached to the end of the cystoscope. The saline was introduced into the empty bladder and aspirated and reintroduced on 3-5 occasions (Hermansen et al 1988). Approximately 30-50mls of fluid were retrieved at the end of the procedure and emptied into 2 sterile containers. The samples were then spun down by centrifugation at 2000 rpm for 10 mins

and the supernatant discarded. The cellular pellets were resuspended in 1 ml of phosphate buffered saline and transferred to 2ml cryotubes. These were centrifuged for 5 mins at 1000 rpm and the supernatant again discarded. The resulting pellets from each washout were then stored in liquid nitrogen until assayed.

The procedure of collecting bladder washouts is very similar to washouts performed routinely by the surgeons either prior to tumour resection in the presence of bleeding from the tumour to improve operative visibility, or at the completion of resection to evacuate resected tumour. For this reason specific patient consent was not obtained as materially no extra procedures other than those routinely performed were being undertaken.

2.3.6 Bladder Tumour Specimens

A sample of fresh tumour was stored, if available, in patients with bladder tumours from whom a bladder washout specimen had been obtained. When appropriate the bladder tumour was resected transurethrally via a cystoscope by the surgeon. At the end of the procedure the bladder was drained and then evacuated in the conventional manner and the resected tissue separated by sieving. Macroscopically representative pieces of tumour were stored immediately in cryotubes in liquid nitrogen. The histology of the tissue stored was assumed to be similar to that reported by the pathologists in the subject's case records. If no tumour was available for the purposes of this study DNA was extracted from paraffin embedded tissue.

2.4 Methods

2.4.1 DNA Extraction

DNA was extracted from cultured cells, breast and bladder tissue, bladder washing samples and nipple aspirate fluid samples using the QIAamp tissue kit (Qiagen). The reagents proteinase k (17.8mg/ml), buffer ATL, buffer AL and buffer AW were all included in the kit and were prepared following the manufacturers instructions. The composition of buffers ATL, AL and AW was not indicated by the manufacturers. The principle of the method is that following cell lysis nucleic acids are selectively absorbed on to a silica-gel based membrane prior to elution with an appropriate buffer. The procedure was performed following the manufacturers instructions and is briefly described below.

2.4.1a DNA Extraction from Cultured cells

DNA was extracted from approximately 10^7 cultured cells. The frozen pellet of cells was brought to room temperature and 200 μ l of phosphate buffered saline, 25 μ l of proteinase k (17.8mg/ml) and 200 μ l of buffer AL were added. The sample was mixed immediately by vortexing and incubated at 70°C for 10 mins. Ethanol (210 μ l) was added and the sample again mixed by vortexing, prior to loading onto a QIAamp spin column in a 2ml collecting tube which was centrifuged at 800 rpm for 1 min. The collection tube was discarded and the spin column placed in a clean collection tube. Wash buffer AW (500 μ l) was added to the spin column which was centrifuged at 800rpm for 1 min. The collection tube was again

discarded, the spin column placed in a 3rd collection tube and a further 500µl of buffer AW added. The column was centrifuged at 800rpm for 1 min and then for a further 2 mins at 1400 rpm, to remove traces of buffer. Finally the spin column was placed in a clean 1.5ml microfuge tube and the DNA eluted by the addition of 200µl of 10mmol Tris HCl pH 9.0 preheated to 70°C to the spin column and centrifugation at 800rpm for 1 min. The purity and concentration of the DNA were assessed by absorbance spectrophotometry at 260 and 280nm. The concentration of the DNA was adjusted to approximately 100ng/µl.

2.4.1b DNA Extraction from Frozen Tissue (Breast and Bladder)

DNA was extracted from approximately 25mg aliquots of tissue (breast or bladder) cut from the frozen samples that had been stored in liquid nitrogen. The tissue was cut into small pieces with a clean scalpel blade, thawed and placed in a 1.5ml microfuge tube to which 180µl of buffer ATL was added. 20µl of proteinase k (17.8mg/ml) was added and the sample mixed by vortexing, followed by incubation at 55°C until the tissue had completely lysed (typically 1-3 hours). Following the incubation buffer AL (200µl) was added, mixed by vortexing and the sample incubated at 70°C for 10 mins. Ethanol (210µl) were then added to the sample, which was mixed and the sample loaded on to a spin column. Subsequent steps were performed as described above.

2.4.1c DNA Extraction from Paraffin embedded tissue (Breast and Bladder)

Paraffin embedded tissue blocks were obtained from the Pathology Department at the Western General Hospital. DNA was extracted as follows using the protocol suggested by the manufacturers for paraffin embedded tissue. Paraffin embedded tissue (25mg) was placed in a 2ml microfuge tube and xylene (1200µl) added. The sample was vortexed thoroughly, and the sample centrifuged at 1400 rpm. for 5 minutes. The supernatant was removed by pipetting without disturbing the tissue pellet and 1200µl of ethanol was added to remove residual xylene. The sample was centrifuged for 5 minutes at 1400 rpm. and the ethanol supernatant removed by pipetting. Further ethanol (1200µl) was added, the sample recentrifuged and the supernatant removed once more. The tube was left open and incubated at 37°C for 10 minutes to enable residual ethanol to evaporate. Subsequently buffer ATL (180 µl) was added and the remaining steps in the procedure were performed as for the frozen tissue method, except that the DNA sample was eluted in 50µl of 10mmol Tris HCl pH 9.0 rather than 200µl because of the lower yield of DNA.

2.4.1d DNA Extraction from Bladder Washing Specimens

DNA was extracted from bladder washing specimens using the same protocol as for extraction from cultured cells except that the final DNA elution was made with 50µl of 10mmol Tris HCl pH 9.0.

2.4.1e DNA Extraction from Nipple Aspirate Samples

DNA was extracted from nipple aspirate samples by the method recommended by the manufacturers for extraction of DNA from small samples of blood, cerebrospinal fluid, or bone marrow dried on microscope slides. This is essentially the method described for cultured cells except that the nipple fluid sample was initially thawed then dissolved in 180 μ l of phosphate buffered saline and the final DNA elution was in 30- 50 μ l of 10mmol Tris HCl pH 9.0 depending on initial sample size.

2.4.1f Further Methods of DNA Preparation from Nipple Aspirate Fluid Samples.

In addition (as described in Chapter 5 section 5.5) 2 other methods of preparation of nipple aspirate fluids for PCR amplification were evaluated. In the first method approximately 5 μ l of nipple aspirate fluid was placed in a Eppendorf tube, with no additional preparation and the PCR reaction mix added; the rationale being that for a simple cellular sample dispersed in a body fluid the temperature cycling of a PCR reaction was all that was required to release DNA into solution to act as the template for the reaction to proceed. This method had previously been described as having being used successfully on nipple aspirate fluid (Motomura et al 1994). Secondly a modification of a method originally described for the PCR amplification of single cell samples such as sperm was used (Li et al 1988, Kovach et al 1991). In this method cell lysis was performed by the additon of a lysis buffer to the nipple aspirate sample

(releasing DNA into solution) in an Eppendorf tube prior to the addition of the other PCR reagents. Briefly: 5 µl of nipple aspirate fluid was added to a microfuge tube along with 2µl of 10x PCR reaction buffer (Promega), 4µl 0.1M DTT, 0.6µl of proteinase K (17.8mg/ml) and 2µl of 17µM SDS made up to 20µl with sterile distilled water. This was incubated at 37⁰C for 1 hour and then 85⁰C for 5 minutes. The 20µl of solution was then used as the template for a 100µl PCR reaction.

2.4.2 Polymerase Chain Reaction

2.4.2a Polymerase Chain Reaction Oligonucleotide Primers

Four pairs of primers were designed, each to amplify individually the whole of either exon 5,6,7 or 8 of the p53 gene. The primers were derived from the intronic areas flanking the areas of interest (enabling the whole of the exon to be amplified) using sequence x54156 for the human p53 gene obtained from the European Molecular Biology Laboratory Genbank database (sequence submitted by Chumakov PM.). The sequence of exons 5-8 and flanking introns is shown in Appendix 2. All primers were 20mers and had the following sequences:

Exon 5	sense	5' TGTGCCCTGACTTTCAACTC
	antisense	5' AACCAGCCCTGTCGTCTCTC
Exon 6	sense	5' TGATTCCTCACTGATTGCTC
	antisense	5' ACCCCAGTTGCAAACCAGAC

Exon 7	sense	5' AAGGCGCACTGGCCTCATCT
	antisense	5' CAGTGTGCAGGGTGGCAAGT
Exon 8	sense	5' GGACCTGATTTCCTTACTGC
	antisense	5' GAGGCATAACTGCACCCTTG

The above primer pairs gave products of the following lengths:

Exon 5 263 base pairs, the 5' end of the sense primer being 42 bases upstream from the 5' end of exon 5 and the 5' end of the antisense primer being 37 bases downstream of 3' end of the exon.

Exon 6 157 base pairs, the 5' end of the sense primer being 24 bases upstream from the 5' end of exon 6 and the 5' end of the antisense primer being 20 bases downstream of 3' end of the exon.

Exon 7 181 base pairs, the 5' end of the sense primer being 40 bases upstream from the 5' end of exon 7 and the 5' end of the antisense primer being 30 bases downstream of 3' end of the exon.

Exon 8 241 base pairs, the 5' end of the sense primer being 50 bases upstream from the 5' end of exon 8 and the 5' end of the antisense primer being 54 bases downstream of 3' end of the exon.

All primers were synthesised by the Imperial Cancer Research Fund Central Services Oligonucleotide Synthesis Facility. They were supplied dry and were prepared for use by removing sideproducts of the synthesis process as follows. The dried pellet was dissolved in 200µl 0.3M sodium

acetate, 10mM MgCl₂ and 600µl cold ethanol added. The mixture was left overnight at -20°C, centrifuged and washed with 80% cold ethanol prior to reconstitution in sterile distilled water. The concentration was assessed by spectrometry at 260nm, and adjusted as appropriate to 200ng/ml.

2.4.2b Polymerase Chain Reaction

The polymerase chain reaction was used to amplify specific exons of the p53 gene. The methods used were based on those described by Saiki (1990). Reactions were performed either in 20µl or 100µl total volume depending on the purpose of the reaction. When the PCR was performed to optimise reaction conditions for each primer pair, or to generate PCR product to be used for direct sequencing a 100µl reaction was performed. When the purpose of the reaction was to generate a radiolabelled product for SSCP analysis a 20µl volume was used (primarily to minimise the amount of radioactivity used). In experiments investigating the the amplification of DNA from nipple aspirate samples both reaction volumes were employed depending on the precise nature of the individual experiment as described in Chapter 5.

For a 100µl standard PCR reaction 1µl of DNA (at approximately 100ng/µl) was added to each reaction tube. A reaction master mix was prepared on ice containing appropriate multiples of the following reagents:

10µl of 10x PCR reaction buffer

2µl dNTPs at 10mM

1mM MgCl₂ (4μl of 25mM stock solution)

1μl of each of the appropriate pair of primers (200ng/ml)

80.5μl of distilled water

0.5μl (2.5units) of PIC/Taq DNA polymerase

The master mix was mixed by vortexing and centrifuged to eliminate bubbles. 99μl were added to each reaction tube. Following mixing and centrifugation 100μl of light mineral oil was added to each tube to prevent evaporation during thermal cycling. In each PCR experiment a tube containing distilled water instead of DNA template was used as a negative control to ensure the absence of contaminants in the reaction. This tube was prepared last. PCR reactions were performed on a Techne PHC 3 Thermal Cycler (Techne). Cycling conditions are described below. For a 20μl reaction the procedure was as described above save that no mineral oil was used as these reactions were all performed on a Techne PHC 3 Thermal Cycler with a heated lid. The master mix was again prepared on ice and contained appropriate multiples of the following reagents depending on the number of samples being studied:

2μl of 10x PCR reaction buffer

2μl dNTPs at 2mM

1mM MgCl₂ (0.8μl of 25mM stock solution)

0.2μl of each of the appropriate pair of primers (200ng/μl)

13.5μl of distilled water

0.3μl (1.5 units) of PIC/Taq DNA polymerase

19 μ l of the master mix were added to each reaction tube containing 1 μ l of DNA (at approximately 100ng/ μ l).

The same cycling conditions were used as for 100 μ l reactions (see below). When the reaction was radiolabelled for subsequent SSCP analysis 0.2 μ l (0.074 MBq) of Redivue α -³²P dCTP was added per sample to the master mix with a corresponding reduction in the volume of distilled water.

2.4.2c Polymerase Chain Reaction Cycling Conditions

The following cycling conditions were used for both 20 μ l and 100 μ l PCR reactions:

An initial denaturing step at 94⁰C for 7 mins, followed by 35 cycles of 1 min at 94⁰C to denature the DNA, 1 min at 53⁰C (exon 6) , 57⁰C (exons 5 and 8) or 59⁰C (exon 7) to allow primer annealing, and 1 min at 72⁰C for product elongation and then a final 10 min period at 72⁰C.

2.4.3 Single Stranded Conformational Polymorphism (SSCP)

2.4.3a Single Stranded Conformational Polymorphism Analysis Using Non-Denaturing Polyacrylamide Gel Electrophoresis

The following methods are based on those previously published (Orita et al 1989b, Glavac and Dean 1993). Initially a 20 μ l PCR reaction was performed, labelled with ³²P α -dCTP using approximately 100ng of sample DNA as the template. Following this the PCR product (2 μ l) were

mixed with SSCP loading dye (10 μ l) and the resultant sample mixed, microfuged and heated to 95 $^{\circ}$ C for 5 minutes then immediately chilled on ice prior to loading on the gel for electrophoresis. This procedure of heating and chilling in the presence of the formamide in the dye was undertaken to denature the double stranded PCR product into 2 single strands. The chilled sample (4-8 μ l) was loaded into a square toothed well of a non-denaturing polyacrylamide gel. All gels were 0.4mm thick, and the glass plates prepared for gel casting by washing in detergent, drying, wiping with ethanol, and then wiping with dimethyldichlorosilane solution (aiding plate separation following electrophoresis).

Gels of the following composition were used:

- 8% acrylamide with 2% bisacrylamide in 1x TBE with no glycerol
- 8% acrylamide with 2% bisacrylamide in 1x TBE with 10% glycerol
- 10% acrylamide with 1.3% bisacrylamide in 1x TBE with no glycerol
- 10% acrylamide with 1.3% bisacrylamide in 1x TBE with 10% glycerol

Temed (65 μ l) and freshly prepared 10% ammonium persulphate (325 μ l) were added to the gel solution immediately prior to casting the gel to promote polymerisation.

Gels containing glycerol were either run at 10W constant power at room temperature, or at 40W constant power at 4 $^{\circ}$ C. Gels without glycerol were run at 40W constant power at 4 $^{\circ}$ C. All gels were pre-run at the appropriate power for at least 15 minutes before sample loading to ensure

the gel was at uniform temperature. The sample loading wells were irrigated with electrophoresis buffer to remove unpolymerised acrylamide, the samples loaded, and the gel run until the first dye front of the SSCP loading dye (bromophenol blue) was at the bottom of the gel; this required electrophoresis for approximately 4 hours for gels without glycerol at 4⁰C, 8 hours for gels with glycerol at 4⁰C and 16 hours for gels with glycerol at room temperature. All electrophoresis was performed using a Life Technologies' model S2 sequencing gel apparatus (Gibco BRL) with 1x TBE in both the upper and lower buffer reservoirs. Following electrophoresis the gel was transferred to Whatman paper and covered with cellophane prior to drying with a gel drier and subsequent autoradiography, using Kodak X-OMAT XAR-5 film.

2.4.3b Single Stranded Conformational Polymorphism Analysis Using MDE Gel Matrix

SSCP was also performed on MDE (mutation detection enhancement) gels. MDE is a polyacrylamide derived matrix that has been reported to offer more reliable mutation detection by SSCP compared to acrylamide gels (Liu and Sommer 1994). The procedure was identical to that described above for polyacrylamide gels except that 0.6x TBE was used in both the upper and lower buffer reservoirs of the electrophoresis apparatus and the following gel compositions and running conditions were used:

0.5 MDE in 0.6x TBE with no glycerol

0.5 MDE in 0.6x TBE with 5% glycerol

Gels were run either at room temperature at 6W constant power or at 4⁰C

at 25W constant power. Gels were again run until the first dye front (bromophenol blue) was at the bottom of the gel; this required electrophoresis for approximately 4 hours for gels without glycerol at 40°C, 8 hours for gels with glycerol at 40°C, 8 hours for gels at room temperature without glycerol and 12-14 hours for gels with glycerol at room temperature.

2.4.4 DNA Sequencing

Direct sequencing was performed with the Sequenase Version 2.0 DNA sequencing kit (Amersham) using a modification of Winship's method (Winship 1989, Prosser et al 1990). All reagents were included in the kit unless otherwise stated. The primers used were those described previously for use in PCR reactions.

Firstly a 100µl PCR reaction was performed to amplify the fragment to be sequenced. The product was subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualised under ultraviolet light to ensure the integrity of the PCR reaction. The product was purified to remove excess or unincorporated reagents and primers using the QIAquick PCR purification Kit (Qiagen), following the manufacturers instructions (Section 2.4.5). The purified product (1-5µl, representing approximately 50ng of DNA) was used as the template for the sequencing reaction.

2.4.4 a Sequencing: Annealing reaction

The annealing reaction was set up comprising 1µl dimethyl sulphoxide (DMSO), 1µl of either the relevant sense or antisense primer, 1-5µl of purified DNA template, 2µl sequenase reaction buffer made up to a final volume of 10µl with distilled water. The resulting mix was placed at 100°C for 2 mins and immediately transferred to a dry ice/methanol bath for primer annealing to take place.

2.4.4b Sequencing: Labelling reaction

The sequenase enzyme was diluted 1+7 with sequenase dilution buffer and the labelling mix diluted 1+15 with distilled water. Termination tubes were set up for each product containing 0.2µl DMSO and 1.8µl of either ddG, ddA, ddT or ddC termination mix.

To the frozen annealing reaction 1µl 0.1M dithiotheitol (DTT), 0.5µl/0.185Mbq ³⁵S-dATP (αS, > 1000Ci/mmol), 2µl diluted labelling mix and 1.5µl diluted sequenase enzyme were added, mixed and incubated at room temperature for 5 mins.

2.4.4c Sequencing: Termination Reaction

Labelling reaction mixture (3.5µl) was added to each of the four tubes containing the 2µl termination mixes, placed at 37°C for 10 minutes and transferred back to room temperature before adding sequencing stop

solution (4 μ l).

All products were sequenced in both the sense and antisense directions, that is using both members of the primer pair in turn. On occasions the resultant sequence contained unreadable compressions, particularly in areas rich in the bases G and C. Under these circumstances the sequencing reaction was repeated substituting dITP for dGTP in the reactions by the use of the appropriate labelling and termination mixes, as supplied by the manufacturer.

2.4.4d Denaturing Gel Electrophoresis of Sequencing Products

Denaturing gel electrophoresis was performed on the sequencing products. Gels (0.4mm thick) were cast (the glass plates having been prepared for gel casting as for SSCP gels) using 65ml of a ready to use sequencing gel mix (6% w/v acrylamide/0.315% bisacrylamide, 7M urea, 1x TBE), to which 65 μ l of Temed and 325 μ l of freshly prepared 10% ammonium persulphate were added immediately prior to casting. Electrophoresis was performed at 35W constant power at room temperature using a model S2 sequencing gel apparatus (Gibco BRL) with 0.4mm thick shark toothed combs and 1x TBE in both the upper and lower buffer reservoirs. The gel was prerun at 35W for at least 15 mins and the wells washed out with the electrophoresis buffer (to remove any unpolymerized acrylamide) prior to loading the samples. The samples were heat denatured at 95⁰C for 5 mins and immediately chilled on ice prior to loading. 4-8 μ l of product were loaded per well. Only intact non-leaking wells were used and lanes containing air bubbles were

avoided. Electrophoresis was performed for 2- 3 hours depending on the product length, the aim being to maximise the length of readable sequence, whilst avoiding loss of bands off the end of the gel and the loss of definition that occurs due to bands being too close to one another. Following electrophoresis the gel was transferred to Whatman paper and covered with cellophane prior to drying with a Gel drier and subsequent autoradiography, using Kodak X-OMAT XAR-5 film.

2.4.5 Purification of PCR Products for Sequencing

PCR products to be used for direct sequencing were purified to remove excess or unincorporated reagents and primers, using the QIAquick PCR purification kit (Qiagen). Buffer PB and buffer PE were included in the kit. The manufacturers instructions were followed and are described briefly: 5 volumes of buffer PB were added to 1 volume of PCR reaction, mixed and applied to a QIAquick spin column placed in a 2ml collection tube. The sample was spun at 1400rpm in a microcentrifuge for 1 minute, the elute discarded and 0.75 ml of buffer PE added to the column which was again centrifuged for 1 minute. The flow through fluid was discarded prior to a further 1 minute spin to remove traces of buffer PE. Finally the spin column was placed in a clean 1.5 ml microfuge tube and the purified product eluted by the addition of 30 μ l of 10 mM Tris-HCl (pH8.5) to the column, which was then centrifuged at 1400 rpm for 1 minute; the elution fluid containing the purified product.

2.4.6 Agarose Gel Electrophoresis

Non-radiolabelled PCR products were visualised by agarose gel electrophoresis. 1.5% agarose gels were used made up in 1x TAE buffer, with 10µl of ethidium bromide (10mg/ml) added per 100ml of gel. PCR products were mixed with DNA loading dye (5:1), prior to loading on to the gel (5µl or 20µl of PCR product being used depending on the well size of the gel poured). The gels were run in 1x TAE buffer at 100-160 V depending on gel size for approximately 40 minutes (allowing the first dye front to migrate approximately 5-7 cm). A 100 base pair ladder was run in 1 lane to permit the product size to be assessed. Following electrophoresis the gel was visualised using an ultra-violet light box and if a permanent record was required of the gel a polaroid photograph was taken. Electrophoresis was performed using DNA Sub-Cell or Wide Mini Sub-Cell apparatus (Biorad) depending on the gel size required.

2.4.7 Extraction of PCR Products from Gels

2.4.7a Extraction of PCR Products from 1.5% Agarose Gels

On occasions it was necessary to extract a PCR product from a 1.5% agarose gel for subsequent use. A QIAquick gel extraction kit (Qiagen) was used following the manufacturers instructions. Buffer QX1 and buffer PE were supplied with the kit. The method is described briefly. The band of interest was visualised under ultra-violet light and excised from the gel using a clean scalpel blade. The gel slice was weighed in a 1.5 ml tube and 3 volumes of buffer QX1 were added per volume of gel. The sample was incubated at 50°C for 20 minutes and 1 gel volume of isopropanol added.

The sample was added to a spin column placed in a 2ml collection tube and centrifuged at 1400 rpm for 1 minute. The flow through was discarded and 0.5 ml of buffer QX1 added to the column which was again spun in the same manner. The flow through was discarded and 0.75 ml of buffer PE added. The column was centrifuged twice for 1 minute, the flow through being discarded between occasions. The spin column was then placed in a clean 1.5 ml microfuge tube and the purified product eluted by the addition of 50µl of 10 mM Tris-HCl (pH 8.5) to the column, which was centrifuged at 1400 rpm for 1 minute; the elution fluid containing the purified product.

2.4.7b Elution of Single Stranded DNA from Dried SSCP Gels

Samples demonstrating a an SSCP pattern suggestive of a p53 gene mutation were sequenced. If the sample appeared to contain predominantly the mutant form sequencing was performed on PCR product purified from a 100µl PCR reaction. However if the mutant form was present in low proportion compared to wild type a suitable abnormally migrating SSCP band was excised from the SSCP gel for elution and subsequent reamplification prior to sequencing. The gel was marked with several dots of radiolabelled ink prior to autoradiography. The autorad was developed and the gel and film aligned using the ink markers before being stapled together. The single stranded DNA band of interest was identified and excised from the gel. The fragment of gel was placed in a 1.5ml microfuge tube and sterile distilled water (100µl) was added. The fragment was disrupted by maceration using a sterile disposable pipette tip and the sample left overnight on a rotator at 40°C to

elute. The elute (10-20 μ l) was used as a single stranded template for a 100 μ l PCR reaction. The resultant product was subsequently purified and sequenced as described in section 2.4.4.

2.4.8 Cell Lines and Tissue Culture

2.4.8a Cell lines

Human cancer cell lines with known normal and abnormal p53 mutations were identified for use in developmental and dilutional experiments.

Cell lines with normal p53 : 2 cell lines were identified from the literature with a normal p53 sequence, firstly MCF-7 a breast cancer cell line has been shown to have a normal sequence for p53 cDNA from exons 4-9 (Casey et al 1991) and A2780 an ovarian cancer cell line has a normal p53 sequence covering the entire coding region of the gene (Brown et al 1993).

Cell lines with p53 point mutations : human cancer cell lines carrying p53 point mutations were identified from a database housed at the European Molecular Biology Laboratory (Hollstein et al 1994).

Cell line	Origin	Mutation
exon 5 (Hensel et al 1991)		
H69	Small cell lung cancer	codon 171 GAG-TAG
exon 6 (Nigro et al 1989)		
T47D	Breast cancer	codon 194 CTT-TTT
exon7 (Yaginuma et al 1992)		
OVCAR 3	Ovarian cancer	codon 248 CGG-CAG
exon 8 (Bartek et al 1990a)		
MDA MB231	Breast cancer	codon 280 AGA-AAA

None of the above 4 cell lines carrying point mutations have been shown to carry a wild type copy of the affected exon, that is their DNA can be considered to be a template for amplification of a pure source of mutant sequence for the relevant exon.

2.4.8b Tissue Culture

The breast cancer cell lines MCF-7, MDA MB 231, and T47 D were grown in Dulbecco's modified Eagle' medium supplemented with 10% heat inactivated foetal calf serum. The ovarian cancer cell lines A2780 and OVCAR 3, and the small cell lung carcinoma cell line H69 were grown in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum. All cells were maintained at 37⁰C in a humidified incubator with 5% CO₂/ 95% air. Pellets containing 10⁷ cells were prepared and stored in

cryotubes in a -80⁰C freezer. Pellets for each cell line were prepared as were mixtures of each of the mutant carrying cell lines (H69, OVCAR 3, MDA MB 231, and T47 D) with both of the cell lines containing wild type p53 (A2780 and MCF-7) such that in each case the mutation carrying line was present in ever decreasing concentrations : 50%, 30%, 20%, 15%, 10%, 5% and 1%. Cell counting was performed on a Coulter Counter and for the cell line mixtures counting was performed in quadruplicate. All the tissue culture, cell counting and cell mixing was performed by Mr. D. Burns.

CHAPTER 3

ESTABLISHMENT AND OPTIMIZATION OF SINGLE STRANDED CONFORMATIONAL POLYMORPHISM ANALYSIS FOR THE DETECTION OF P53 MUTATIONS IN SHED TUMOUR MATERIAL

Objectives: These were to :

- 1) design and optimise PCR primers to amplify reliably the entire coding sequence of each of exons 5,6,7 and 8 of the p53 gene.
- 2) identify one or more cell lines with a wild type p53 gene sequence and for each exon of interest to identify a cell line carrying a mutation in that exon (Chapter 2.4.8a).
- 3) confirm the p53 status of those cell lines identified by direct sequencing.
- 4) use DNA from the cell lines to optimise SSCP conditions for mutation detection and subsequently to define the sensitivity of SSCP to identify the presence of a mutation in a mixed cell population (i.e. in mixtures of wild type and mutant carrying cells). This latter objective was of fundamental importance to the studies on clinical material as both nipple aspirate fluid and bladder washing samples were likely to contain substantial proportions of normal cells of non-tumour origin.

3.1 Optimisation of PCR reaction conditions

As described in Materials and Methods (section 2.4.2a), 4 pairs of PCR primers were designed to amplify the whole of exons 5, 6,7, and 8 of the p53 gene. Using standard reaction methods, the 2 major variables that need optimisation for each pair of primers are the annealing temperature and the magnesium concentration. The annealing temperature is determined by the length of the oligonucleotide primer and the ratio within the primer sequence of guanosine plus cytidine (which pair in

double stranded DNA with 3 hydrogen bonds) to adenosine plus thymidine (2 hydrogen bonds). Whilst formulae exist that can predict primer annealing temperatures, in practice they require to be determined empirically. Magnesium concentration can affect many aspects of the PCR reaction, including primer annealing, polymerase enzyme activity and DNA strand dissociation temperature (Innis and Gelfand 1990).

Commonly useful concentrations vary between 1 and 2 mmol final concentration in the PCR reaction (Innis and Gelfand 1990). Using a standard 100 μ l PCR reaction 4 Mg²⁺ concentrations (1, 1.25, 1.5 and 2 mmol) were assessed for each primer pair, at each of the following annealing temperatures: 51⁰C, 53⁰C, 55⁰C, 57⁰C and 59⁰C, in addition exon 7 was assessed at 61⁰C, all other reagent concentrations and reaction parameters being fixed. Optimal conditions were defined as those in which a single intense band of PCR product of the appropriate size could reproducibly be visualised following agarose gel electrophoresis.

Illustrative examples of the effect of annealing temperature and Mg²⁺ concentration on reactions are shown in Figure 3.1. It can be seen that for both exon 5 and 6 PCR products single bands of product of the appropriate size (263 and 157 base pairs respectively) are visualised at a Mg²⁺ concentration of 1mmol. However for higher concentrations of 1.5 and 2 mmol non-specific extra bands are seen. Additionally for exon 6 reactions it can be seen that the yield of PCR product (indicated by the intensity of the band of product visualised on agarose gel electrophoresis) is greater when an annealing temperature of 53⁰C rather than 57⁰C is used.

For all 4 pairs of primers a Mg^{2+} concentration of 1mmol was optimal (with higher Mg^{2+} concentrations frequently giving rise to nonspecific extra bands of product). The following annealing temperatures were adopted for subsequent use:

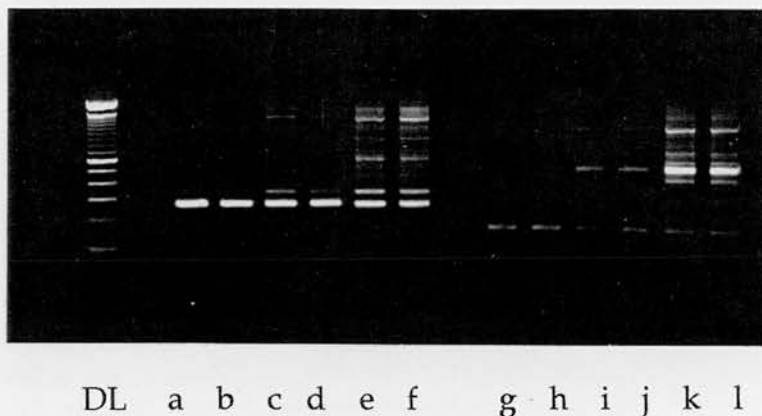
Exon 5 primer pair	57 ⁰ C
Exon 6 primer pair	53 ⁰ C
Exon 7 primer pair	59 ⁰ C
Exon 8 primer pair	57 ⁰ C

The same annealing temperatures and Mg^{2+} concentrations were equally suitable for 20 μ l PCR reactions.

Figure 3.1

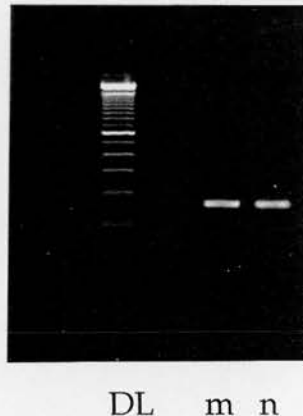
Agarose gel electrophoresis demonstrating the effect of Mg^{2+} concentration and annealing temperature on PCR reactions.

a) Annealing Temperature $57^{\circ}C$.



Lanes a-f represent pairs of samples of exon 5 PCR products with PCR Mg^{2+} concentrations of 1, 1.5 and 2 mmol respectively, and lanes g-l represent pairs of exon 6 PCR products with PCR Mg^{2+} concentrations of 1, 1.5 and 2 mmol respectively. DL represents a 100 base pair DNA ladder.

b) Annealing Temperature $53^{\circ}C$.



Lanes m and n represent a pair of exon 6 PCR products with a PCR Mg^{2+} concentrations of 1 mmol, all reaction conditions being the same as used in Figure 3.1a except for the annealing temperature. DL represents a 100 base pair DNA ladder.

3.2 p53 Status of Cancer Cell Lines Used

The cell lines used are described in the materials and methods chapter (2.4.8a). The p53 status of each of the cell lines used was established by direct sequencing of the exons of interest.

The sequence of exons 5,6,7 and 8 of cell lines MCF-7 and A2780 was confirmed to be wild type (gels not shown; sequence listed in Appendix 2). DNA from these cell lines was therefore used as a source of control wild type DNA in subsequent studies.

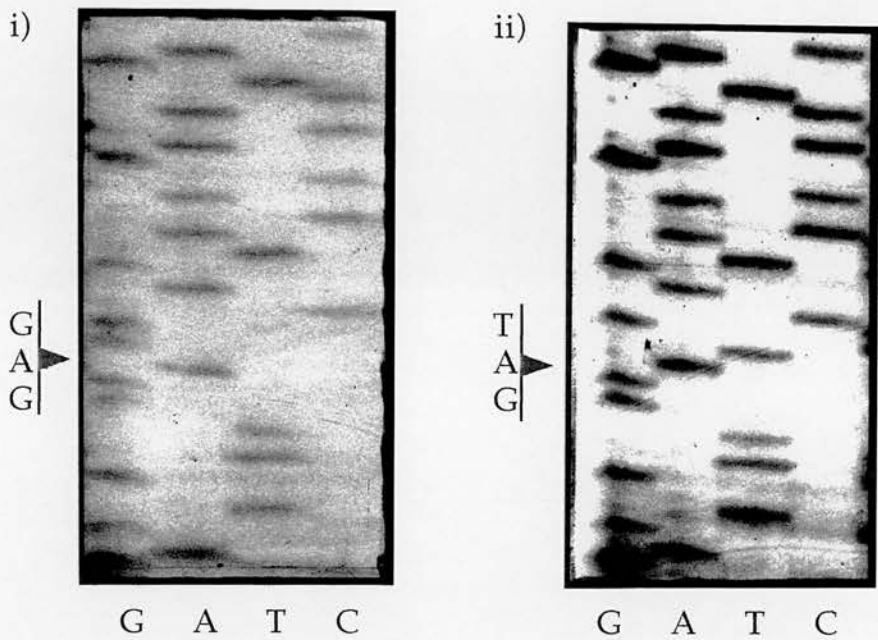
Figure 3.2 demonstrates mutations found in cell lines H69, T47D , OVCAR 3 and MDA MB 231, alongside examples of corresponding wild type sequence. The mutations are listed below and confirm those reported previously (Hensel et al 1991, Nigro et al 1989, Yaginuma et al 1992, Bartek et al 1990a):

H69	exon 5	codon 171	GAG -TAG
T47D	exon 6	codon 194	CTT - TTT
OVCAR 3	exon 7	codon 248	CGG - CAG
MDA MB231	exon 8	codon 280	AGA- AAA

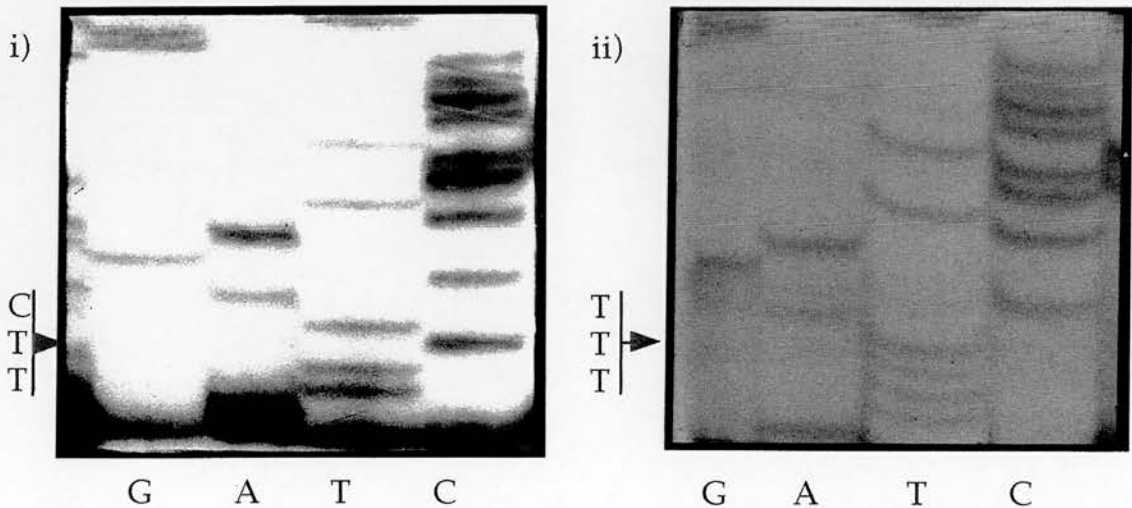
It was confirmed that no additional mutations were present in the exons studied, and that both from sequencing gels and subsequent SSCP patterns none of the cell lines carried a wild type copy of the relevant exon in addition to the mutated one. These cell lines were therefore used as sources of control mutant DNA in subsequent studies.

Figure 3.2
 Autoradiographs of sequencing gels demonstrating the presence of p53 mutations in cancer cell lines H69, T47D, OVCAR 3, and MDA MB 231.

a) i) Cell line A2780 carries a wild type GAG sequence at codon 171 in exon 5 of the p53 gene, whilst ii) cell line H69 is seen to have a GAG - TAG mutation at this site.

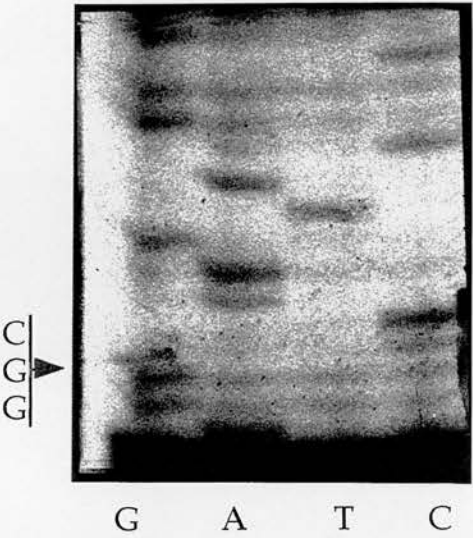


b) i) Cell line A2780 carries a wild type CTT sequence at codon 194 in exon 6 of the p53 gene., whilst ii) cell line T47D is seen to have a CTT - TTT mutation at the same site.

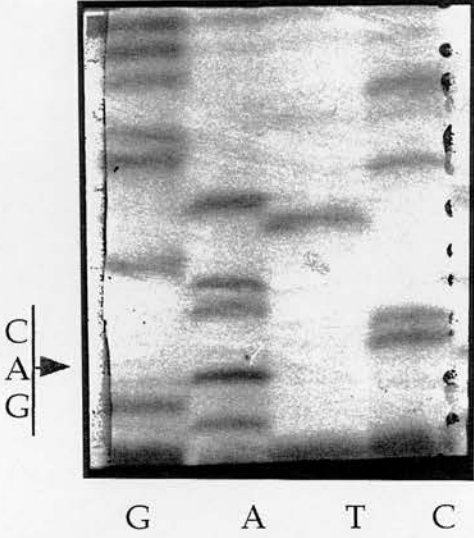


c) i) Cell line A2780 carries a wild type CGG sequence at codon 248 in exon 7 of the p53 gene, whilst ii) cell line OVCAR 3 is seen to have a CGG-CAG mutation at this site.

i)

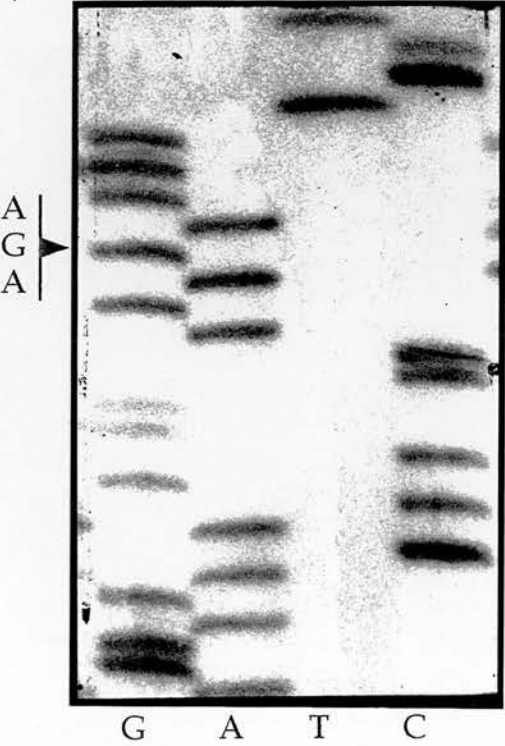


ii)

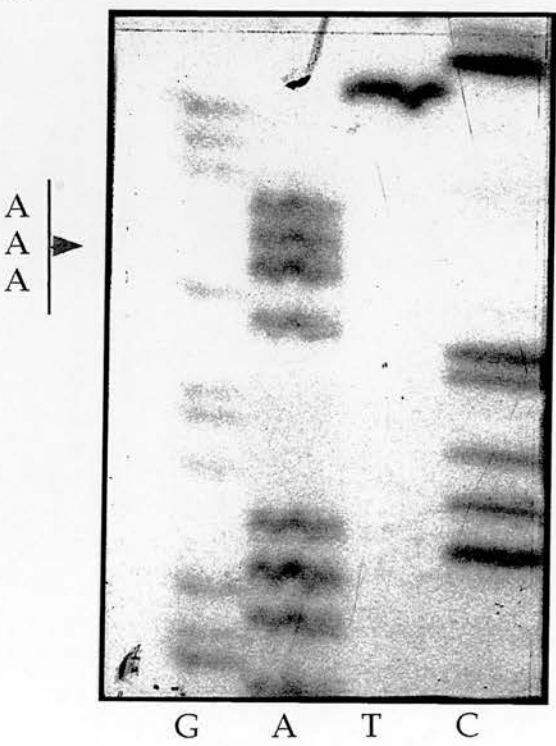


d) i) Cell line A2780 carries a wild type AGA sequence at codon 280 in exon 8 of the p53 gene, whilst ii) cell line MDA MB 231 is seen to have an AGA-AAA mutation at this site.

i)



ii)



3.3 Assessment of the Effect of SSCP Gel Conditions on Distinguishing Wild Type and Mutant PCR Products Using Polyacrylamide Gels

The gel composition and running conditions are known to be of critical importance in the ability of SSCP to demonstrate the presence of a mutation. The technique was originally described using a 5% acrylamide gel at room temperature. However as discussed in Chapter 1.9 reports suggest that modifications such as the addition of 5-10% glycerol to the gel, running the gel at 40°C or the use of a higher concentration of acrylamide (8-10%) with a low concentration of bisacrylamide (1.3-2%) may enhance mutation detection. The addition of glycerol is felt to be particularly important at room temperature. It has been stressed that for optimal mutation detection the product to be screened should be run under 2 or more sets of gel conditions. Taking these data into account the ability of SSCP to infer the presence of a mutation in the 4 known mutation carrying cell lines was assessed under a variety of gel conditions. The gel conditions assessed were as follows:

- A) 8% acrylamide, 2% bisacrylamide with 10% glycerol run at room temperature (8G,RT)
- B) 10% acrylamide, 1.3% bisacrylamide with 10% glycerol run at room temperature (10G,RT)
- C) 8% acrylamide, 2% bisacrylamide with no glycerol run at 40°C (8NG,C)
- D) 10% acrylamide, 1.3% bisacrylamide with no glycerol run at 40°C (10NG,C).
- E) 8% acrylamide, 2% bisacrylamide with 10% glycerol run at 40°C (8G,C)

F) 10% acrylamide, 1.3% bisacrylamide with 10% glycerol run at 40°C (10G,C)

For each exon ³²P radiolabelled PCR was performed using duplicate samples of DNA from each of the 2 wild type cell lines and the mutation carrying line. The demonstration of a difference between samples was only considered to have been successful when there was an unequivocal SSCP pattern difference between the wild type and mutant samples. In instances when the migration pattern was similar/equivocally different the samples were considered to be indistinguishable by SSCP under those particular running conditions. Samples were run on at least 2 separate occasions using products from separate PCR reactions. Examples of autoradiographs are shown in Figure 3.3 and the results summarised in Table 3.1.

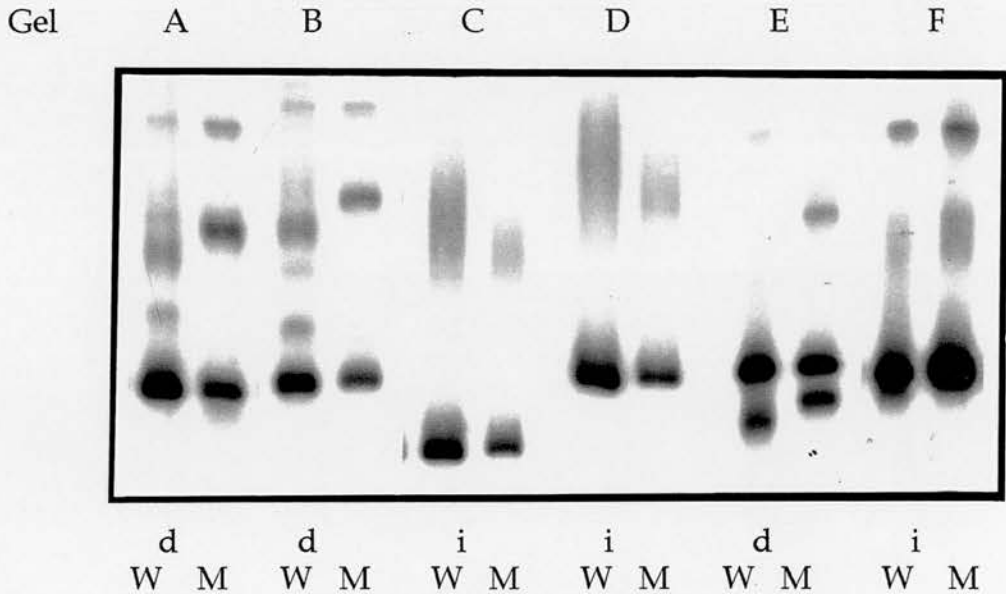
It can be seen that for all mutation carrying cell lines an abnormal SSCP pattern was seen under at least 3 sets of gel conditions, but that only 1 mutation (OVCAR 3, exon 7) was clearly identifiable under all conditions studied. In all cases the SSCP patterns of both the wild type cell line DNA samples (MCF-7 and A2780) were indistinguishable (data not shown). It is seen that on occasions there are several single strand bands visualised rather than the 2 expected. This is a common occurrence with SSCP and is explained by the ability of any given single strand to adopt more than 1 conformation under SSCP conditions, with each conformation having its own migration pattern. It is also noteworthy that, whilst the results appeared reproducible in that if an abnormal SSCP pattern appeared for a particular set of gel conditions repeat testing would again demonstrate a

difference compared to wild type, gels run at room temperature did show a degree of variability in the exact pattern probably reflecting the effect of differences in ambient room temperature on the conformation adopted by the individual single strands.

Figure 3.3

Autoradiographs of SSCP gels demonstrating the effect of gel running conditions on the ability of SSCP to distinguish wild type from mutation carrying PCR products. Gel types: **A** = 8% acrylamide, 2% bisacrylamide with 10% glycerol run at room temperature, **B** = 10% acrylamide, 1.3% bisacrylamide with 10% glycerol run at room temperature, **C** = 8% acrylamide, 2% bisacrylamide with no glycerol run at 40°C, **D** = 10% acrylamide, 1.3% bisacrylamide with no glycerol run at 40°C, **E** = 8% acrylamide, 2% bisacrylamide with 10% glycerol run at 40°C, **F** = 10% acrylamide, 1.3% bisacrylamide with 10% glycerol run at 40°C. Lanes labelled **W** carry wild type product and **M** carry mutant product. **d** indicates that wild type and mutant product were considered distinguishable on SSCP and **i** that they were indistinguishable or equivocally different.

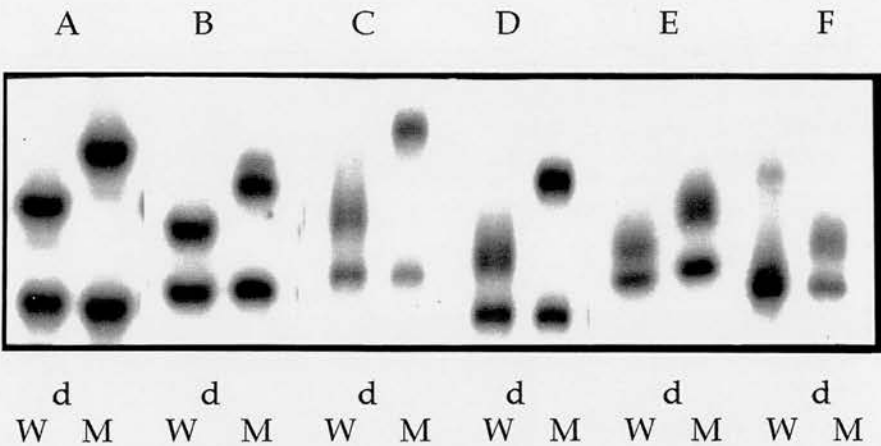
a) Exon 5: comparing SSCP patterns for wild type PCR product and mutation carrying product from H69 cell line.



b) Exon 6: comparing SSCP patterns for wild type PCR product and mutation carrying product from T47D cell line.



c) Exon 7: comparing SSCP patterns for wild type PCR product and mutation carrying product from OVCAR 3 cell line.



d) Exon 8: comparing SSCP patterns for wild type PCR product and mutation carrying product from MDA MB 231 cell line.

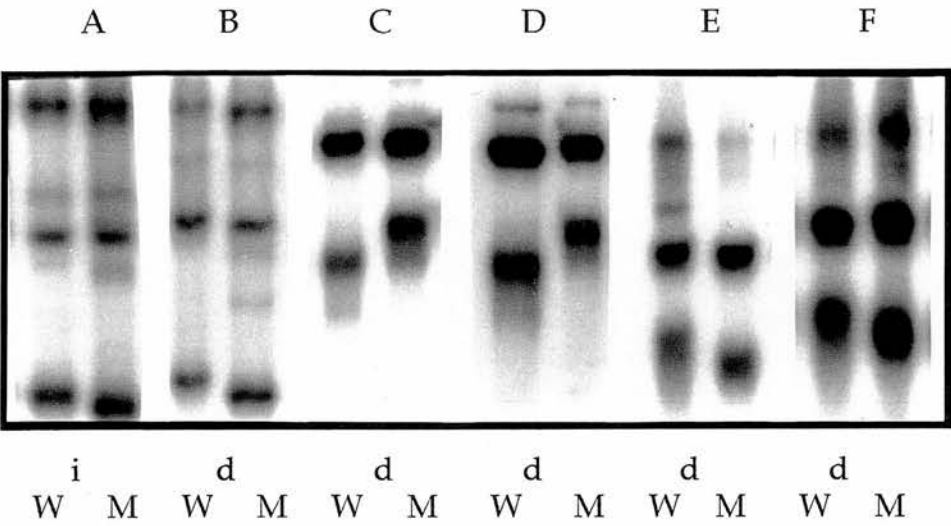


Table 3.1

The effect of polyacrylamide gel running conditions on the ability of SSCP to detect a difference between wild type PCR product (WT) and products carrying mutations in exons 5, 6, 7 or 8 of the p53 gene (H69, T47D, OVCAR 3, MDA MB 231 respectively). **DIST** indicates that mutant and wild type were clearly distinguishable and **IND** that they were indistinguishable. Gel type is as defined in section 3.3.

	<u>Exon 5</u>	<u>Exon 6</u>	<u>Exon 7</u>	<u>Exon 8</u>
<u>Gel</u>	<u>H69/WT</u>	<u>T47D/WT</u>	<u>OVCAR3/WT</u>	<u>MDA /WT</u>
A: 8G,RT	DIST	DIST	DIST	IND
B: 10G,RT	DIST	DIST	DIST	DIST
C: 8NG,C	IND	IND	DIST	DIST
D: 10NG,C	IND	DIST	DIST	DIST
E: 8G,C	DIST	IND	DIST	DIST
F: 10G,C	IND	IND	DIST	DIST

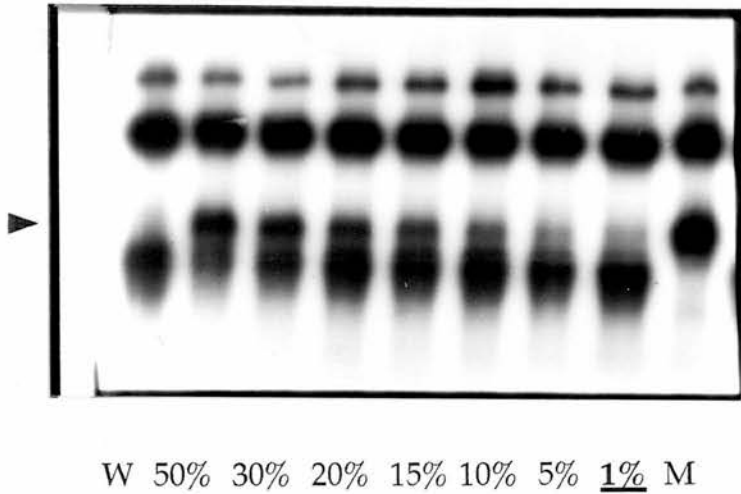
3.4 Sensitivity of SSCP in Mutation Detection in a Mixed Cell Population Using Polyacrylamide Gels

It was regarded as being of central importance to the subsequent clinical studies to assess the sensitivity of SSCP to detect mutations in a mixture of mutation-carrying and wild type cells. This would model the nature of the clinical samples to be collected (which from their nature, would be likely to contain such a mixture). Previous work had suggested that mutations could be detected using SSCP in mixtures containing a lower limit of between 5% and 25% of mutation carrying cells (Smith TA et al 1992, Wu J et al 1993). Accordingly duplicate series of cell dilutions were prepared for each mutation carrying cell line containing a range from 1%-50% of mutant cells made up with MCF-7 or A2780 cells respectively. PCR-SSCP was performed on DNA from each of the dilution series on those gel types that had earlier been shown to demonstrate that particular mutation. The MCF-7 and A2780 series were run in parallel, so that in effect the experiments were performed in duplicate. Representative examples are illustrated in Figure 3.4 and the results summarised in Table 3.2. It is seen that the range of limits of sensitivity obtained was wide (range 1-30% of mutation carrying cells in the sample), but that for each of the 4 mutations studied 1 or more sets of gel conditions yielded a most sensitive detection limit of 5% or fewer mutant cells. It is noteworthy that on the occasions when the results between the A2780 and MCF series differed, in each case, greater sensitivity was seen with the A2780 series.

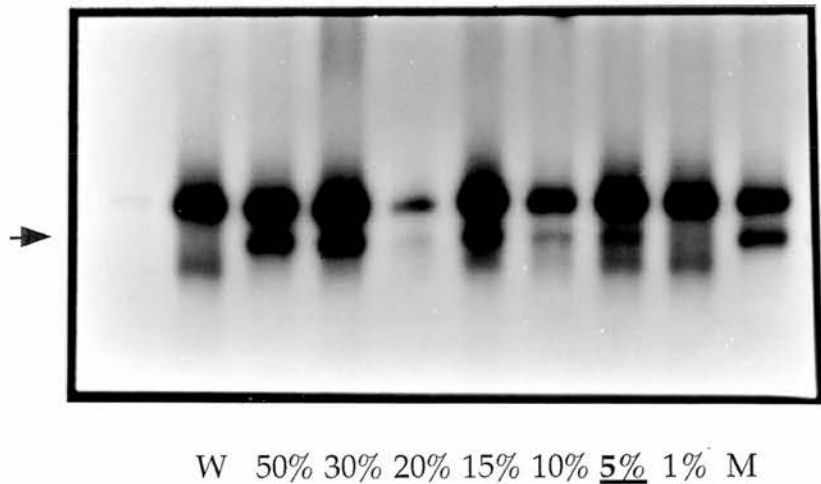
Figure 3.4

Autoradiographs of SSCP performed on polyacrylamide gels demonstrating examples from the range of sensitivities of mutation detection in DNA extracted from mixtures of wild type and mutation carrying cells. M denotes mutant product, W wild type and X% indicates the percentage of mutation carrying cells in samples. Arrows indicate abnormally migrating mutant bands.

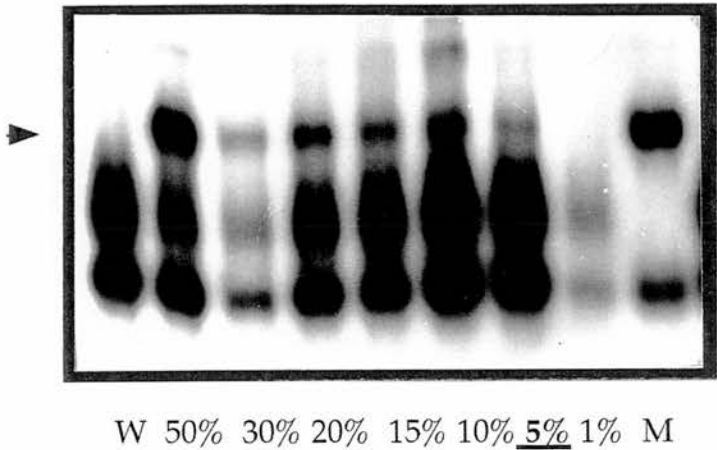
a) SSCP of p53 exon 8 PCR products run on an 8% acrylamide gel without glycerol at 4⁰C. The presence of an abnormal SSCP pattern remains discernable in mixtures of cells containing 1% of mutation carrying cells.



b) SSCP of p53 exon 5 PCR products run on an 8% acrylamide gel with glycerol at 4⁰C. The presence of an abnormal SSCP pattern is discernable in mixtures of cells containing 5% or more mutation carrying cells.



c) SSCP of p53 exon 7 PCR products run on a 10% acrylamide gel without glycerol at 40°C. The presence of an abnormal SSCP pattern is discernable in mixtures of cells containing 5% or more mutation carrying cells.



d) SSCP of p53 exon 8 PCR products run on an 8% acrylamide gel with glycerol at 40°C. The presence of an abnormal SSCP pattern is discernable in mixtures of cells containing 30% or more mutation carrying cells.

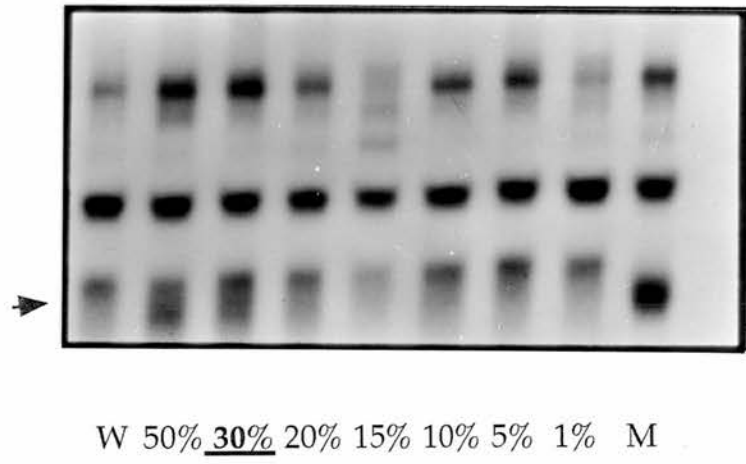


Table 3.2

The effect of SSCP gel running conditions on the ability of SSCP to demonstrate the presence of mutation carrying DNA in the presence of wild type DNA. For each gel condition used the sample containing the lowest percentage of mutation carrying cells in which an abnormal SSCP pattern could be discerned is indicated. Gel type is as defined in section 3.3

Exon 5: dilutions of H69 cells with p53 wild type A2780 and MCF 7 cells

<u>Gel</u>	<u>A2780 series</u>	<u>MCF-7 series</u>
A: 8G,RT	5%	10%
B: 10G,RT	5%	10%
E: 8G,C	5%	5%

Exon 6: dilutions of T47 D cells with p53 wild type A2780 and MCF 7 cells

	<u>A2780 series</u>	<u>MCF-7 series</u>
A: 8G,RT	30%	30%
B: 10G,RT	30%	30%
D:10NG,C	5%	10%

Exon 7: dilutions of OVCAR 3 cells with p53 wild type A2780 and MCF 7 cells

	<u>A2780 series</u>	<u>MCF-7 series</u>
A: 8G,RT	5%	10%
B: 10G,RT	5%	10%
C: 8NG,C	5%	10%
D: 10NG,C	5%	10%
E: 8G,C	20%	30%
F: 10G,C	20%	20%

Exon 8 dilutions of MDA MB 231 cells with p53 wild type A2780 and MCF 7 cells

	<u>A2780 series</u>	<u>MCF-7 series</u>
A: 10G,RT	30%	30%
C: 8NG,C	1%	1%
D: 10NG,C	10%	10%
E: 8G,C	20%	30%
F: 10G,C	20%	30%

3.5 SSCP Using MDE Gel Matrix

The use of a novel polyacrylamide-derived matrix, MDE (Mutation Detection Enhancement) has been reported to significantly improve the ability of SSCP to resolve subtle conformational differences between samples, with resultant increased sensitivity in terms of mutation detection (Liu and Sommer 1994). It was therefore felt appropriate to investigate whether these properties may also offer an advantage in terms of the sensitivity of mutation detection in a mixed cell population. A variety of gel conditions have been described for MDE, and for the purposes of this study the following were assessed, taking into account the manufacturers recommendations:

A: 0.5x MDE in 0.6x TBE with no glycerol run at room temperature (MNG,RT)

B: 0.5 MDE in 0.6x TBE with 5% glycerol run at room temperature (MG,RT)

C: 0.5 MDE in 0.6x TBE with no glycerol run run at 4⁰C (MNG,C)

D: 0.5 MDE in 0.6x TBE with 5% glycerol run at 4⁰C (MG,C)

Two series of experiments were performed, the first to establish which gel conditions were able to demonstrate a difference in SSCP pattern between wild type PCR product and that of each of the 4 mutation carrying cell lines and the second to establish the limits of detection for SSCP when mutant and wild type cell mixes were analysed.

3.5.1 Assessment of the Effect of SSCP Gel Conditions on Distinguishing Wild Type and Mutant PCR Products Using MDE Gels

For each exon ^{32}P radiolabelled PCR-SSCP was performed using samples of DNA from a wild type cell line and the mutation carrying line. The demonstration of a difference between samples was only considered to have been successful when there was an unequivocal SSCP pattern difference between the wild type and mutant samples. In instances when the migration pattern was similar/equivocally different the samples were considered to be indistinguishable by SSCP under those particular running conditions. Samples were run on at least 2 separate occasions using products from separate PCR reactions, results in all cases being concordant. The effect of gel type on the SSCP pattern for exon 8 samples is illustrated in Figure 3.5, and the results are summarised in Table 3.3. For each mutation an abnormal SSCP pattern was seen under at least 2 sets of conditions, with the exon 7 mutation seen under all 4 and therefore in this small series MDE gels would appear to offer no particular advantage to the polyacrylamide gels tested earlier. The addition of glycerol to MDE gels would appear to improve the likelihood of mutation detection, with a difference between wild type and mutant being seen in 7 out of 8 occasions for glycerol containing gels and 3 out of 8 for gels without glycerol. However the sample size is too small to apply meaningful statistical analysis.

Figure 3.5
 Autoradiographs of SSCP gels demonstrating the effect of MDE gel running conditions on the ability of SSCP to distinguish wild type from mutation carrying exon 8 PCR products. Gel types: A = 0.5x MDE gel with no glycerol run at room temperature, B = 0.5x MDE gel with 5% glycerol run at room temperature, C = 0.5x MDE gel with no glycerol run at 4⁰C, D = 0.5x MDE gel with 5% glycerol run at 4⁰C. Lanes labelled W carry wild type product and M carry mutant product. **d** indicates that wild type and mutant product were considered distinguishable on SSCP and **i** that they were indistinguishable or equivocally different.

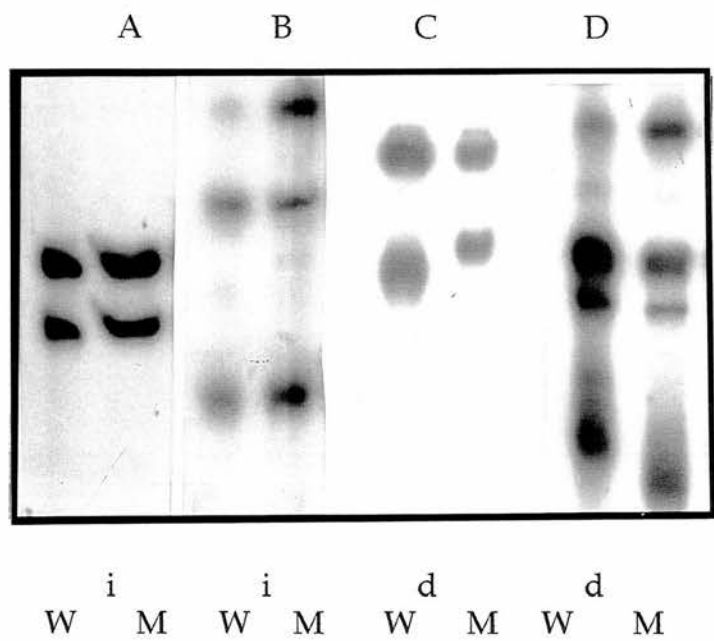


Table 3.3

The effect of MDE gel running conditions on the ability of SSCP to detect a difference between wild type PCR product (WT) and products carrying mutations in exons 5, 6, 7 or 8 of the p53 gene (H69, T47D, OVCAR 3, MDA MB 231 respectively). **DIST** indicates that mutant and wild type were clearly distinguishable and **IND** that they were indistinguishable.

	<u>Exon 5</u>	<u>Exon 6</u>	<u>Exon 7</u>	<u>Exon 8</u>
<u>Gel</u>	<u>H69/WT</u>	<u>T47D/WT</u>	<u>OVCAR3/WT</u>	<u>MDA /WT</u>
A: MNG,RT	IND	IND	DIST	IND
B: MG,RT	DIST	DIST	DIST	IND
C: MNG,C	IND	IND	DIST	DIST
D: MG,C	DIST	DIST	DIST	DIST

3.5.2 Sensitivity of SSCP in Mutation Detection in a Mixed Cell Population Using MDE Gels

The primary objective of this series of experiments was to establish whether or not SSCP performed using a variety of MDE gel conditions was superior in sensitivity to polyacrylamide gel analysis. Consequently DNA from the cell mixes described earlier were used, but this time using only the A2780 series of mixes in the 1%-20% range. Each series was run on each of the gel conditions able to demonstrate the mutation in that particular exon on 2 occasions using products from the same PCR

reaction. Autoradiographs illustrating gels from throughout the range of sensitivities detected are shown in Figure 3.6. The results are summarised in Table 3.4 below, showing in each case the lower limit of detection for each dilution series, in terms of percentage of mutation carrying cells in the sample. NA indicates that for that particular mutation and gel type the sensitivity was not assessed because mutant and wild type forms were indistinguishable. Where results differed between the 2 runs the second result is shown in brackets.

Table 3.4

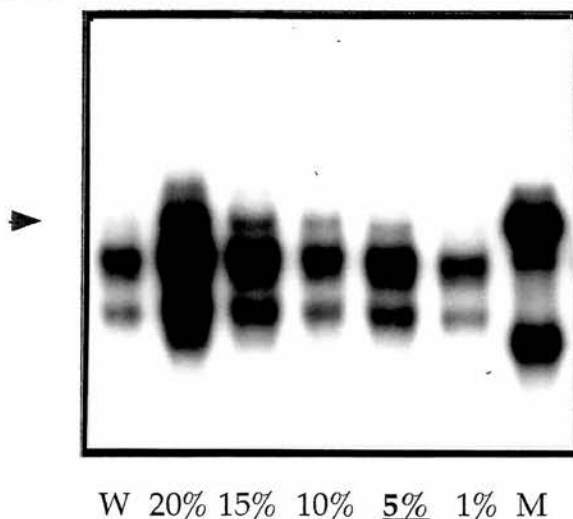
	Exon 5	Exon 6	Exon 7	Exon 8
Cell Mix	H69/WT	T47 D/WT	OVCAR 3/WT	MDA/WT
Gel Type				
A: MNG, RT	NA	NA	5%	NA
B: MG, RT	5%	>20%	15%	NA
C: MNG,C	NA	NA	5%	10%
D: MG,C	5%	>20%	15% (10%)	>20%

It can be seen that as for acrylamide gels there is substantial variability in the sensitivities observed. The lower limit of detection was 5% mutation carrying cells for 2 of the mutations studied. However for the T47D / Wild Type mix the mutation was not detectable at the 20% level for either of the gel conditions studied, and for the MDA/ Wild Type mix the detection limit was 10%. The use of MDE gels for SSCP under the conditions studied appeared therefore to offer no advantage in terms of sensitivity over acrylamide gels.

Figure 3.6

Autoradiographs of SSCP performed on MDE gels demonstrating examples from the range of sensitivities of mutation detection in DNA extracted from mixtures of wild type and mutation carrying cells. **M** denotes mutant product, **W** wild type and **X%** indicates the percentage of mutation carrying cells in samples. Arrows indicate abnormally migrating mutant bands.

a) SSCP of p53 exon 7 PCR products run on a 0.5x MDE gel without glycerol at room temperature. The presence of an abnormal SSCP pattern is discernable in mixtures of cells containing 5% or more mutation carrying cells.



b) SSCP of p53 exon 8 PCR products run on a 0.5x MDE gel without glycerol at 4°C. The presence of an abnormal SSCP pattern is discernable in mixtures of cells containing 10% or more mutation carrying cells.

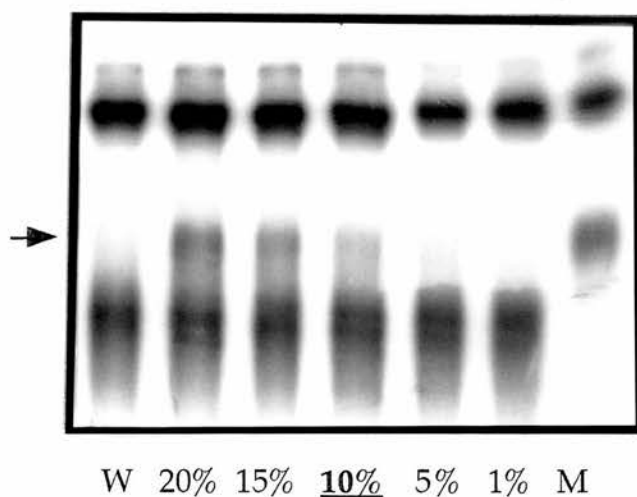
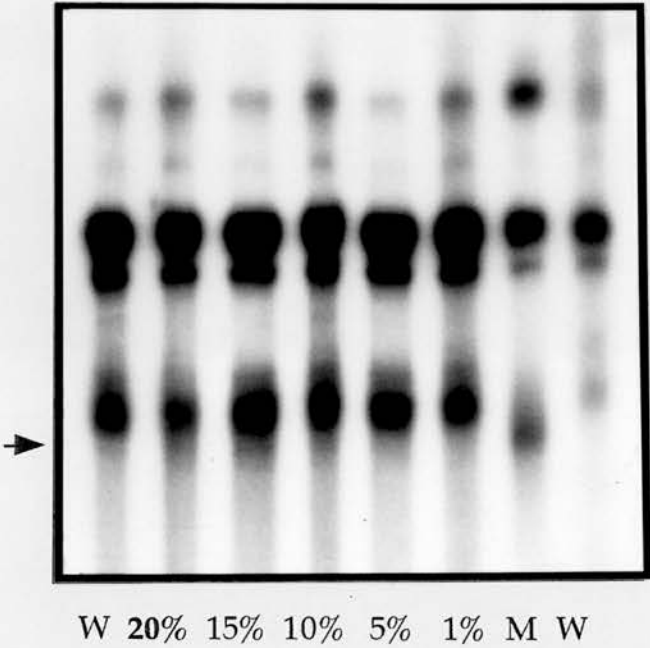


Figure 3.6
c) SSCP of p53 exon 8 PCR products run on a 0.5x MDE gel with 5% glycerol at 40C. The presence of an abnormal SSCP pattern is not discernable even in mixtures containing 20% mutation carrying cells.



Discussion

The above data illustrate a number of important points:

- 1) The ability of SSCP to detect the presence of a mutation depends critically on the gel conditions used.
- 2) The sensitivity of SSCP in detecting the presence of mutations in a mixed cell population also depends critically on the gel conditions used.
- 3) Since in only one instance an abnormal SSCP pattern was seen for a sample containing only 1% of mutation carrying cells, this is likely to be the best limit of sensitivity achievable with this technique. This was not tested by performing further dilutions as in the context of the other results even if a lower level had been found it would not have been typical of that commonly achievable.
- 4) When using polyacrylamide, a lower detection limit of 5% or better was achieved for each of the 4 A2780/mutant dilution series, the optimal gel conditions being variable and unpredictable. This level could be regarded as a realistic practical limit of detection.
- 5) MDE gels appeared to offer no particular advantage over polyacrylamide gels, either in terms of distinguishing mutant from wild type PCR products, or in terms of the ability to detect SSCP abnormalities in samples containing only a small proportion of mutation carrying cells. Thus as the MDE gel matrix is considerably more expensive than acrylamide it was not further used.
- 6) When there was a sensitivity difference between the A2780 and MCF-7 dilution series this was always in the same direction (the detection limit being lower in the A2780 series). The reasons for this are not clear though it could either reflect a systematic error in counting of one of the cell lines or possibly a difference in p53 gene copy number between the 2 cell

lines. The differences were never more than to the next dilution level and as such did not materially affect the conclusions or implications of the results.

It can be concluded that to maximise mutation detection in a mixed cell population SSCP has to be performed on at least 2 or 3 different sets of gel conditions because the sensitivity of any particular gel type for a particular mutation can not be predicted. There have been only 2 previous reports of studies addressing this issue. Wu et al (1993) performed SSCP on mixtures of wild type DNA and tumour DNA from 2 tumours with p53 point mutations. In one case the detection limit was 5% and in the other 15%. Smith et al (1992) describe a study mixing cells with and without a heterozygous mutation of the CTP synthetase gene. The mutation was identifiable by SSCP in mixes containing 25% of mutation carrying cells. This latter study's results are not directly comparable to those investigating p53 mutations, as the CTP synthetase mutation carrying cells also carried one normal copy of the gene, further diluting the effective proportion of the mutation, whereas in the case of a p53 mutation the second allele is most commonly deleted. However even if this is accounted for, the limits of detection are not as good as in the present study. It is perhaps noteworthy that both the above studies would appear to have used only 1 set of SSCP conditions, emphasising the importance of using multiple conditions if sensitivity is to be maximised. There are little published data on the sensitivity of other mutation detection screening methods in this context, such as dideoxy fingerprinting, heteroduplex analysis, denaturing gradient gel electrophoresis and chemical cleavage of mismatch. However as the limiting factor in all techniques would be the resolution of identifying an

abnormally migrating DNA product on an electrophoresis gel, none are likely to improve significantly on that demonstrated for SSCP. As discussed elsewhere if the objective changes to the detection of previously characterised mutations improvement of several orders of magnitude can be achieved.

It has been reported that the use of MDE gel matrix increases the sensitivity of SSCP in distinguishing wild type and mutant PCR products (Liu and Sommer 1994). This was not demonstrated in the current study, however these findings are consistent with further reports (Ravnik-Glavac et al 1994, Vidal-Puig and Moller 1994) that indicated that fully optimised SSCP using acrylamide gels was as sensitive as with MDE gels. There are no published data on the sensitivity of SSCP in detecting abnormalities in mixed cell populations using MDE, but the current study does not support the use of MDE in this setting.

From a practical point of view a decision had to be taken as to the SSCP gel conditions to be used to analyse the samples from the 2 clinical studies undertaken. Bearing in mind the variability of the results seen it was decided to choose for each exon 1 room temperature gel with glycerol, 1 gel at 40°C with glycerol and 1 at 40°C without glycerol. The gels were chosen largely on the basis of the sensitivity of the gels to demonstrate mutations in cell mixing experiments. However for exon 5 neither of the gels tested without glycerol at 40°C demonstrated an abnormal SSCP pattern for H69 cell line DNA and therefore 1 was chosen randomly; the same applied to exon 6 for T47D cell line DNA and gels with glycerol run at 40°C.

The following gels were used in analysis of clinical samples:

Exon 5	A: 10G,RT	C: 8NG,C	E: 8G,C
Exon 6	A: 10G,RT	C: 8NG,C	E: 8G,C
Exon 7	A: 10G,RT	D: 10NG,C	E: 8G,C
Exon 8	A: 10G,RT	D: 10NG,C	E: 8G,C

It is fully appreciated that the sensitivities of SSCP elicited in the mixing experiments will not apply to all mutations in that particular exon, as the nature of the mutation will influence SSCP migration patterns and thus possibly sensitivity. However the sensitivities were used as a pragmatic guide, in the absence of more reliable guides to making a rational choice.

CHAPTER 4

STUDIES ON p53 MUTATIONS IN BLADDER WASHING SPECIMENS FROM PATIENTS WITH BLADDER CANCER

Aims: The objective of this part of the study was to investigate whether it was possible to detect the presence of malignancy in bladder washing samples from patients with known bladder cancer, using p53 mutations as a biochemical marker. Previously published work based on a small series of patients had indicated that the presence of p53 mutations was detectable in the urine of such patients by using time consuming methodologies involving cloning and the use of mutation specific oligonucleotide probes designed for the particular mutation earlier identified in that subject's tumour (Sidransky et al 1991).

For the purposes of this study mutations in p53 were screened for using SSCP, which as has been discussed earlier is a comparatively simple mutation detection method, not requiring prior characterisation of the particular mutation present.

The DNA yield from bladder washing specimens was found to be such that it was feasible to perform multiple PCR reactions on DNA extracted from each specimen (data not shown). Therefore the strategy of investigation was first to screen the bladder washing samples for the presence of mutations and subsequently to confirm their presence in the corresponding bladder tumour sample whilst also screening the tumour samples for the presence of any mutations not detected in the bladder washings. Any samples found on SSCP to have an abnormal pattern suggestive of the presence of a mutation were subjected to direct DNA

sequencing.

Results

4.1 SSCP Analysis of Bladder Washing Specimens

31 bladder washing specimens were collected from a total of 27 patients with known bladder cancer, samples being collected from 4 patients on 2 separate occasions. In all cases there was a macroscopically visible bladder abnormality present at the time the bladder washing was collected. Details of the age of subject, histological type and grade of tumour, pathological stage when known and details of any previous treatment for bladder cancer are shown in Appendix 2. Pathological evaluation of the surgical biopsy from 2 patients with a prior history of bladder cancer revealed no tumour but the presence of CIS in one case and dysplastic urothelium in the other.

DNA was extracted from the bladder washing samples. PCR-SSCP was performed for each of exons 5-8 of the p53 gene using 3 sets of SSCP conditions for each exon as discussed in Chapter 3. For each exon studied the PCR reaction included a negative, no template control to demonstrate absence of contamination of the PCR reaction and a sample both of wild type and mutation carrying cell line DNA for that exon; the wild type sample acting as a normal reference control, to which the migration pattern of the tumour DNA samples could be compared and the mutation carrying sample allowing comparison of migration pattern to that of previous gels run under the same conditions ensuring interassay consistency. Where the migration pattern of a sample indicated the possibility of a p53 mutation a further PCR reaction was performed and

SSCP repeated. If there was a disparity between the 2 results the PCR-SSCP was repeated again and the sample only considered to potentially contain a mutation if the same abnormal migration pattern was seen on at least 2 occasions, the abnormal pattern otherwise being attributed to artifact.

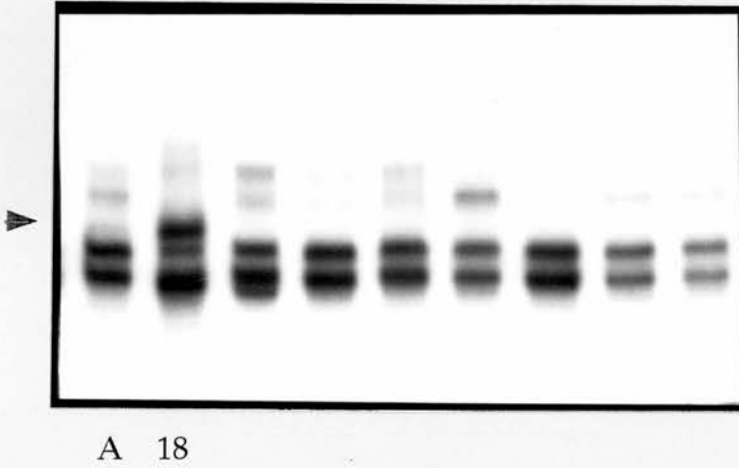
A total of 5 samples from 5 separate patients demonstrated an abnormal SSCP pattern. Two abnormalities were seen in exon 6, 2 in exon 7 and 1 in exon 8, whilst for all subjects the SSCP pattern for exon 5 was indistinguishable from wild type. These abnormalities are demonstrated in Figure 4.1 and the results are summarised in Table 4.1 (exons 5 and 6) and Table 4.2 (exons 7 and 8).

It is noteworthy that of the 5 subjects in whom an abnormal SSCP pattern was demonstrated 1 had 2 bladder washing samples collected at the time of different cystoscopies, with 1 sample demonstrating an abnormality and the other not (samples 18 and 26 respectively; Figure 4.1a,b, Table 4.1). It can also be seen that of the 5 abnormalities found, only 2 were seen under all 3 sets of gel conditions used (samples 18 and 27; Table 4.1), with 1 being seen under 1 (sample 30; Table 4.2) and 2 under 2 sets of conditions (samples 3 and 19; Table 4.2).

Figure 4.1

Autoradiographs of SSCP gels demonstrating abnormal migration patterns consistent with the presence of mutations in 5 out of 31 bladder washing samples from patients with bladder cancer.

a) Exon 6. An abnormal extra band is seen in sample 18 (indicated by arrow); lane A is A2780 cell line normal control; all other lanes contain bladder washing samples with normal SSCP pattern.



b) Exon 6. An abnormal extra band is seen in sample 27 (indicated by arrow); lane A is A2780 cell line normal control; all other lanes contain bladder washing samples with normal SSCP pattern, in particular sample 26 which is from the same patient as sample 18 (Figure 4.1a) but taken on a separate occasion doesn't show an abnormality despite the presence of tumour at cystoscopy at the time of sample collection.

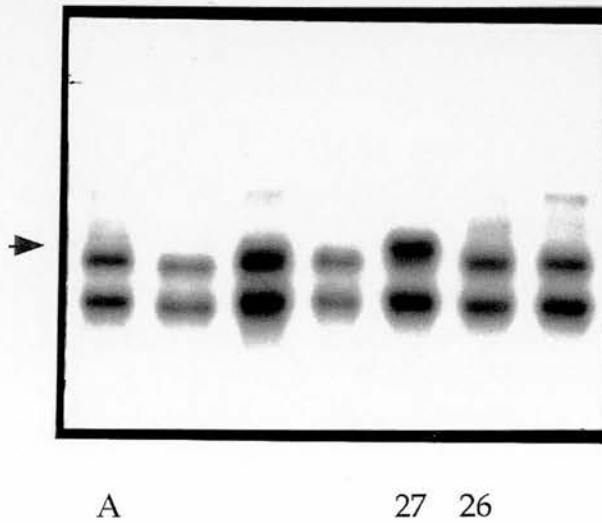
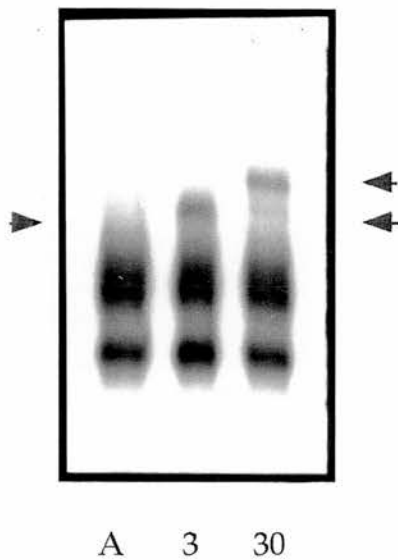


Figure 4.1 c) Exon 7. An abnormal extra band is seen in sample 3 and two extra bands in sample 30 (indicated by arrows); lane A is A2780 cell line normal control.



d) Exon 8. An abnormal extra band is seen in sample 19 (indicated by arrow); lane A is A2780 cell line normal control; all other lanes contain bladder washing samples with normal SSCP pattern.

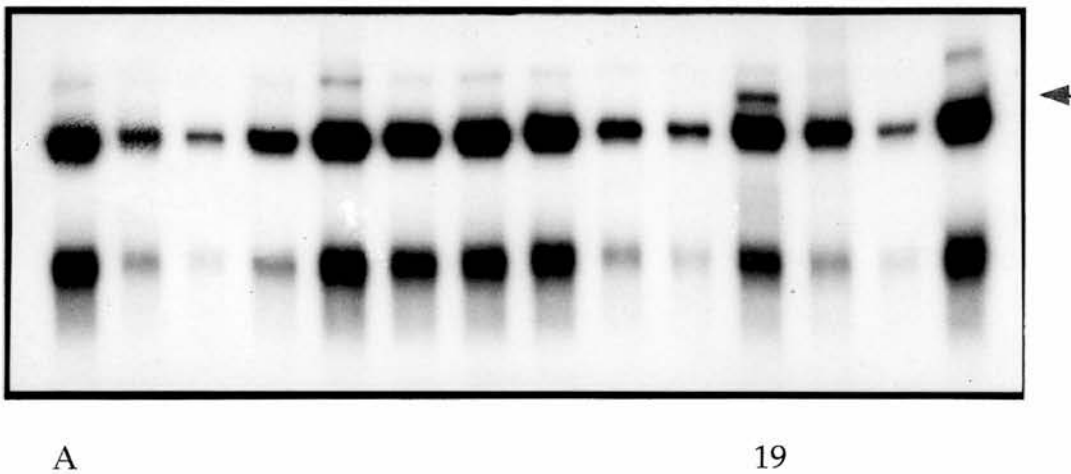


Table 4.1

Results of SSCP analysis of Exons 5 and 6 of the p53 gene in 31 Bladder Washing Samples.

		Exon 5	Exon 5	Exon 5	Exon 6	Exon 6	Exon 6
Patient	Sample	Gel A	Gel C	Gel D	Gel A	Gel C	Gel D
A	3	N	N	N	N	N	N
B	6	N	N	N	N	N	N
C	7	N	N	N	N	N	N
D	8	N	N	N	N	N	N
E1	9	N	N	N	N	N	N
E2	24	N	N	N	N	N	N
F	10	N	N	N	N	N	N
G1	12	N	N	N	N	N	N
G2	33	N	N	N	N	N	N
H	13	N	N	N	N	N	N
I	14	N	N	N	N	N	N
J1	15	N	N	N	N	N	N
J2	31	N	N	N	N	N	N
K	16	N	N	N	N	N	N
L	17	N	N	N	N	N	N
M1	18	N	N	N	ABN	ABN	ABN
M2	26	N	N	N	N	N	N
N	19	N	N	N	N	N	N
O	20	N	N	N	N	N	N
P	21	N	N	N	N	N	N
Q	22	N	N	N	N	N	N
R	23	N	N	N	N	N	N
S	25	N	N	N	N	N	N
T	27	N	N	N	ABN	ABN	ABN
U	28	N	N	N	N	N	N
V	29	N	N	N	N	N	N
W	30	N	N	N	N	N	N
X	32	N	N	N	N	N	N
Y	40	N	N	N	N	N	N
Z	41	N	N	N	N	N	N
AA	50	N	N	N	N	N	N

Patient suffix 1 and 2 indicates samples taken on separate occasions

N : Normal SSCP Pattern

ABN: Abnormal SSCP Pattern

Gel A: 10% acrylamide, 1.3% bisacrylamide, no glycerol, 40°C

Gel C: 8% acrylamide, 2% bisacrylamide, 10% glycerol, 40°C

Gel D: 10% acrylamide, 1.3% bisacrylamide, 10% glycerol,
room temperature

Table 4.2
Results of SSCP analysis of Exons 7 and 8 of the p53 gene in 31 Bladder Washing Samples.

		Exon 7	Exon 7	Exon 7	Exon 8	Exon 8	Exon 8
Patient	Sample	Gel B	Gel C	Gel D	Gel B	Gel C	Gel D
A	3	ABN	N	ABN	N	N	N
B	6	N	N	N	N	N	N
C	7	N	N	N	N	N	N
D	8	N	N	N	N	N	N
E1	9	N	N	N	N	N	N
E2	24	N	N	N	N	N	N
F	10	N	N	N	N	N	N
G1	12	N	N	N	N	N	N
G2	33	N	N	N	N	N	N
H	13	N	N	N	N	N	N
I	14	N	N	N	N	N	N
J1	15	N	N	N	N	N	N
J2	31	N	N	N	N	N	N
K	16	N	N	N	N	N	N
L	17	N	N	N	N	N	N
M1	18	N	N	N	N	N	N
M2	26	N	N	N	N	N	N
N	19	N	N	N	ABN	N	ABN
O	20	N	N	N	N	N	N
P	21	N	N	N	N	N	N
Q	22	N	N	N	N	N	N
R	23	N	N	N	N	N	N
S	25	N	N	N	N	N	N
T	27	N	N	N	N	N	N
U	28	N	N	N	N	N	N
V	29	N	N	N	N	N	N
W	30	ABN	N	N	N	N	N
X	32	N	N	N	N	N	N
Y	40	N	N	N	N	N	N
Z	41	N	N	N	N	N	N
AA	50	N	N	N	N	N	N

Patient suffix 1 and 2 indicates samples taken on separate occasions

N : Normal SSCP Pattern ABN: Abnormal SSCP Pattern

Gel B: 8% acrylamide, 2% bisacrylamide, no glycerol, 40C
Gel C: 8% acrylamide, 2% bisacrylamide, 10% glycerol, 40C
Gel D: 10% acrylamide, 1.3% bisacrylamide, 10% glycerol,
 room temperature

4.2 SSCP Analysis of Bladder Tumour Specimens

Freshly-stored bladder tumour specimens collected at the time of bladder washing sample collection were available for 23 of the 31 washing samples. These included samples from 16 of the 23 subjects from whom 1 bladder washing was collected. Of the 4 subjects from whom 2 washings were collected, fresh tumour was available from 1 of the cystoscopies in 1 case and both in 3 cases. All tumour samples available were screened for the presence of mutations in exons 5,6,7, and 8 of the p53 gene as described for the bladder washing specimens.

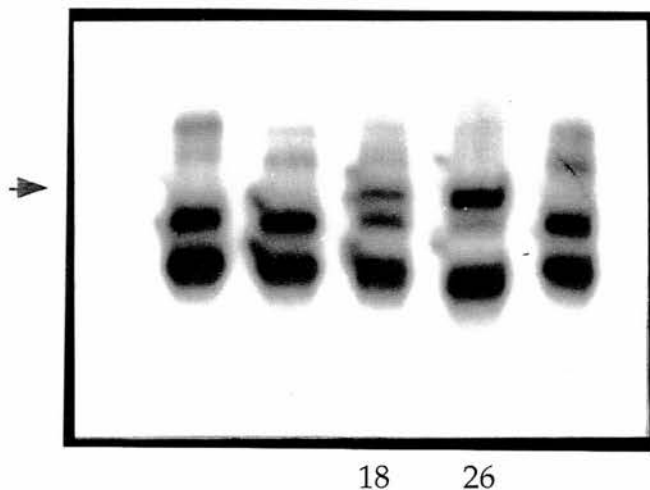
Six tumour specimens from 4 subjects demonstrated an abnormal SSCP pattern. Of the mutations, 1 was in exon 7, 2 in exon 6 and 3 in exon 8. These abnormalities are demonstrated in Figure 4.2 and the results summarised in Table 4.3 (exons 5 and 6) and Table 4.4 (exons 7 and 8). The abnormal samples included 3 tumour specimens from 2 of the 5 patients who had bladder washing samples demonstrating an SSCP abnormality. In these cases the abnormal patterns of the washing and corresponding tumour were of similar appearance, this being highly indicative of the samples carrying the same mutation (an example is demonstrated in Figure 4.2c). There were in addition 3 tumour samples (from 2 patients) with abnormal SSCP patterns in whom the corresponding washing samples were of normal appearance (demonstrated in Figures 4.2b and 4.2c); of these the abnormality from one patient was seen on only 1 gel type and from the other on 2 gel types.

Therefore there were 3 cases (samples 3, 27 and 30) in which the tumour showed no evidence of a mutation but in whom the bladder washing

Figure 4.2

SSCP gels demonstrating abnormal migration patterns consistent with the presence of mutations in the p53 gene in 6 out of 23 bladder tumour specimens.

a) Exon 6. An abnormal extra band (indicated by arrow) is seen in the same position in both samples 18 and 26; these samples being bladder tumour specimens collected from the same patient on 2 separate occasions.



b) Exon 7. An abnormal extra band (indicated by arrow) is seen in sample 41T (bladder tumour). Lane A is an A2780 cell line normal control. Sample 41W is the bladder washing sample corresponding to sample 41T; the SSCP pattern is indistinguishable from the normal control with no extra band visualised.

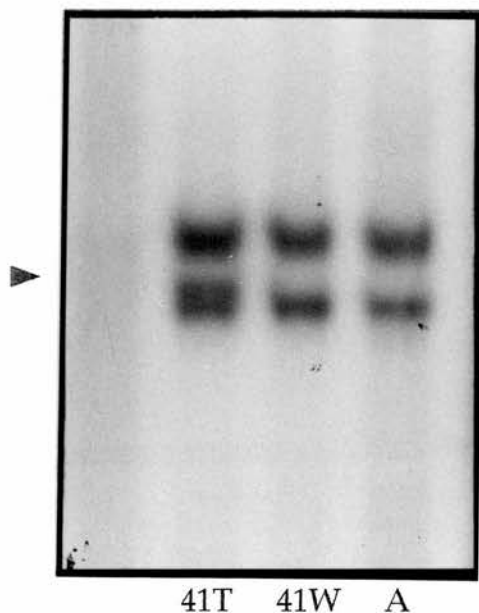


Figure 4.2 continued.

c) Exon 8. An abnormal extra band (indicated by arrow) is seen in the same position in both samples 9T and 24T; these samples being bladder tumour specimens collected from patient E on 2 separate occasions. However this abnormal extra band is not visualised in either of the corresponding bladder washing samples 9W and 24W. An abnormal extra band (indicated by \blacklozenge) is seen in the same position in samples 19T and 19W representing bladder tumour and bladder washing samples respectively taken on the same occasion from patient N, demonstrating the same abnormality in matched samples. Lane A is an A2780 cell line normal control.

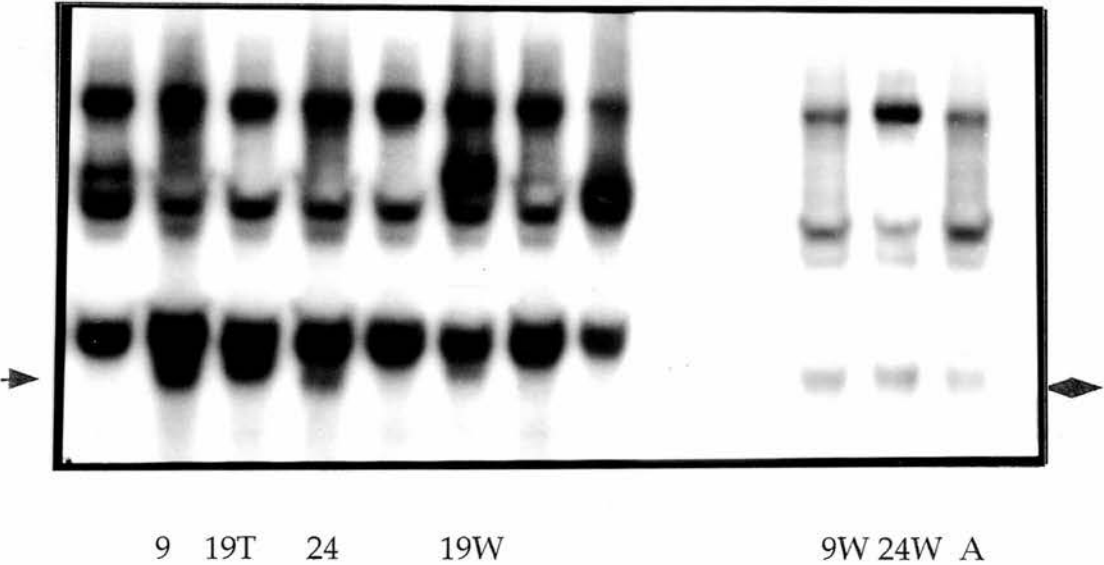


Table 4.3
Results of SSCP analysis of Exons 5 and 6 of the p53 gene in the 23 Bladder Tumour Specimens available that were collected at the same time as corresponding Bladder Washing Samples (no tumour being available on 8 out of 31 occasions).

		Exon 5	Exon 5	Exon 5	Exon 6	Exon 6	Exon 6
Patient	Sample	Gel A	Gel C	Gel D	Gel A	Gel C	Gel D
A	3	N	N	N	N	N	N
B	6	N	N	N	N	N	N
C	7	N	N	N	N	N	N
D	8	N	N	N	N	N	N
E1	9	N	N	N	N	N	N
E2	24	N	N	N	N	N	N
G1	12	N	N	N	N	N	N
G2	33	N	N	N	N	N	N
H	13	N	N	N	N	N	N
I	14	N	N	N	N	N	N
J	15	N	N	N	N	N	N
K	16	N	N	N	N	N	N
M1	18	N	N	N	ABN	ABN	ABN
M2	26	N	N	N	ABN	ABN	ABN
N	19	N	N	N	N	N	N
O	20	N	N	N	N	N	N
Q	22	N	N	N	N	N	N
S	25	N	N	N	N	N	N
T	27	N	N	N	N	N	N
U	28	N	N	N	N	N	N
W	30	N	N	N	N	N	N
Y	40	N	N	N	N	N	N
Z	41	N	N	N	N	N	N

Patient suffix 1 and 2 indicates samples taken on separate occasions

N : Normal SSCP Pattern ABN: Abnormal SSCP Pattern

Gel A: 10% acrylamide, 1.3% bisacrylamide, no glycerol, 4⁰C
Gel C: 8% acrylamide, 2% bisacrylamide, 10% glycerol, 4⁰C
Gel D: 10% acrylamide, 1.3% bisacrylamide, 10% glycerol,
 room temperature

Table 4.4 Results of SSCP analysis of Exons 7 and 8 of the p53 gene in the 23 Bladder Tumour Specimens available that were collected at the same time as corresponding Bladder Washing Samples (no tumour being available on 8 out of 31 occasions).

		Exon 7	Exon 7	Exon 7	Exon 8	Exon 8	Exon 8
Patient	Sample	Gel B	Gel C	Gel D	Gel B	Gel C	Gel D
A	3	N	N	N	N	N	N
B	6	N	N	N	N	N	N
C	7	N	N	N	N	N	N
D	8	N	N	N	N	N	N
E1	9	N	N	N	ABN	N	N
E2	24	N	N	N	ABN	N	N
G1	12	N	N	N	N	N	N
G2	33	N	N	N	N	N	N
H	13	N	N	N	N	N	N
I	14	N	N	N	N	N	N
J	15	N	N	N	N	N	N
K	16	N	N	N	N	N	N
M1	18	N	N	N	N	N	N
M2	26	N	N	N	N	N	N
N	19	N	N	N	ABN	N	ABN
O	20	N	N	N	N	N	N
Q	22	N	N	N	N	N	N
S	25	N	N	N	N	N	N
T	27	N	N	N	N	N	N
U	28	N	N	N	N	N	N
W	30	N	N	N	N	N	N
Y	40	N	N	N	N	N	N
Z	41	ABN	N	ABN	N	N	N

Patient suffix 1 and 2 indicates samples taken on separate occasions

N : Normal SSCP Pattern

ABN: Abnormal SSCP Pattern

Gel B: 8% acrylamide, 2% bisacrylamide, no glycerol, 4⁰C

Gel C: 8% acrylamide, 2% bisacrylamide, 10% glycerol, 4⁰C

Gel D: 10% acrylamide, 1.3% bisacrylamide, 10% glycerol, room temperature

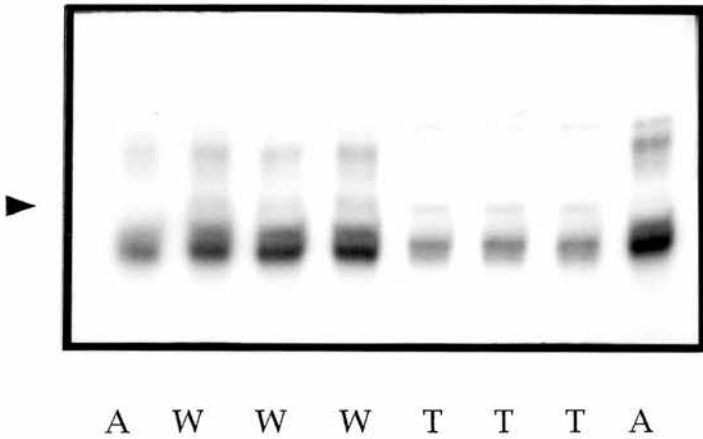
showed an abnormality in p53. The 4 main potential reasons for this are: i) that the abnormalities demonstrated in the washing were artifacts; ii) the tumour specimen taken was not representative of the tumour as a whole; iii) the supposed tumour specimen actually consisted largely of normal tissue despite its macroscopic appearance; and finally iv) the p53 abnormality seen in the washing sample had a source other than the visible bladder tumour.

To attempt to address this issue paraffin embedded bladder tumour specimens were obtained from the pathology department for all 3 subjects in this category, the tumour sample having been obtained at the operation at which the washing specimen was taken. DNA was extracted from these specimens and PCR-SSCP performed for the relevant exon. The results are illustrated in Figure 4.3. It can be seen that in each case an abnormal SSCP pattern is demonstrated for the paraffin embedded tumour sample including abnormal bands comigrating with the abnormal bands seen in the corresponding bladder washing samples. However for tumour samples corresponding to bladder wash 30 (Patient W) only 1 extra band is seen rather than the expected 2; the paraffin blocks from which samples were taken for this patient contained an estimated 20-40% tumour and this level may be the explanation for only 1 of the 2 abnormal bands being seen.

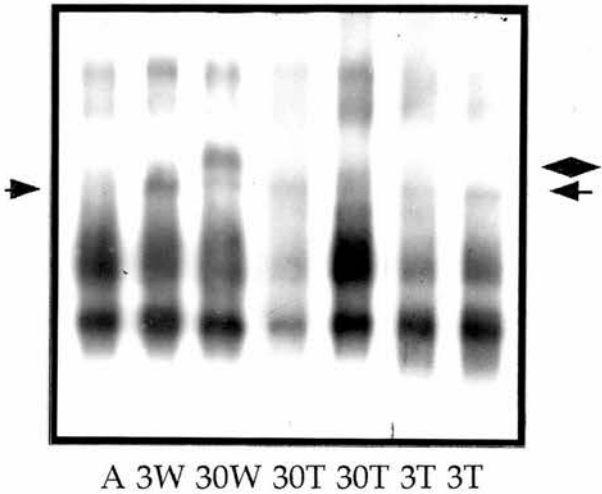
The results indicate that low representation by tumour in the fresh samples studied for these patients was the probable explanation for their normal SSCP appearance.

Figure 4.3
SSCP gels demonstrating the presence of abnormal migration patterns in bladder washing samples and paraffin embedded tissue samples from subjects from whom the fresh sample did not demonstrate a p53 abnormality.

a) Exon 6. Bladder washing samples (W) and paraffin embedded tumour samples (T) in triplicate from patient T. An abnormal extra band (indicated by arrow) is seen in all samples except the A2780 cell line normal controls (A).



b) Exon 7. An abnormal extra band is clearly seen in bladder washing sample 3W (→) and 2 extra abnormal bands in bladder washing sample 30W (← and ◆), when compared to A2780 cell line normal control (A). The corresponding abnormal band is seen in samples of paraffin embedded tumour for sample 3 (labelled 3T). For sample 30 only 1 of the 2 extra bands is seen in paraffin embedded tumour (30T).



4.3 Correlation Between Mutation Detection In Surgical Biopsy/ Resection Samples and Bladder Washing Samples.

The concordance between the SSCP results obtained in tumour tissue and bladder washing samples was evaluated. For the purposes of this correlation a bladder tumour specimen was regarded as containing a mutation if an abnormal SSCP pattern was seen in either fresh tissue or in paraffin embedded tissue. There were 20 patients for whom pairs of tumour and bladder washings were available for study:

16 with 1 bladder washing sample and corresponding tumour

3 with 2 bladder washings and tumour samples from both occasions

1 with 2 bladder washings but tumour from only 1 occasion.

In total therefore there were 23 pairs of samples obtained synchronously.

As demonstrated in Table 4.5, concordant results were obtained for the p53 status of tumour and bladder washing specimens on 19 out of 23 occasions (83%).

A total of 9 tumour samples from 7 patients

demonstrated a p53 abnormality with the abnormality seen in the

corresponding bladder washing sample in 5 cases (56%). In 1 patient 2

pairs of samples showed an abnormality in the tumour samples but

neither of the bladder washings. In 1 case 2 pairs of samples were again

available with only 1 of the 2 bladder washings demonstrating the

abnormality seen in both tumour samples. In the final case an

abnormality detected in tumour was not demonstrable in the

corresponding bladder washing.

Table 4.5

Correlation between SSCP abnormalities in the p53 gene detected in paired Bladder Washing and Tumour Samples. Suffix 1 and 2 indicate samples taken from the patient indicated on separate occasions.

Patient	Sample	Tumour SSCP	Bladder Wash SSCP	Correlate
A	3	Abnormal Exon 7	Abnormal Exon 7	Yes
B	6	Normal	Normal	Yes
C	7	Normal	Normal	Yes
D	8	Normal	Normal	Yes
E1	9	Abnormal Exon 8	Normal	No
E2	24	Abnormal Exon 8	Normal	No
G1	12	Normal	Normal	Yes
G2	33	Normal	Normal	Yes
H	13	Normal	Normal	Yes
I	14	Normal	Normal	Yes
J	15	Normal	Normal	Yes
K	16	Normal	Normal	Yes
M1	18	Abnormal Exon 6	Abnormal Exon 6	Yes
M2	26	Abnormal Exon 6	Normal	No
N	19	Abnormal Exon 8	Abnormal Exon 8	Yes
O	20	Normal	Normal	Yes
Q	22	Normal	Normal	Yes
S	25	Normal	Normal	Yes
T	27	Abnormal Exon 6	Abnormal Exon 6	Yes
U	28	Normal	Normal	Yes
W	30	Abnormal Exon 7	Abnormal Exon 7	Yes
Y	40	Normal	Normal	Yes
Z	41	Abnormal Exon 7	Normal	No

4.4 Sequencing of Mutations Detected

Samples demonstrating an abnormal SSCP pattern were subjected to direct sequencing to characterise the exact nature of the mutation present. There were specimens from 7 patients that had shown an abnormal SSCP pattern in either bladder washing samples, bladder tumour samples or both (Table 4.5).

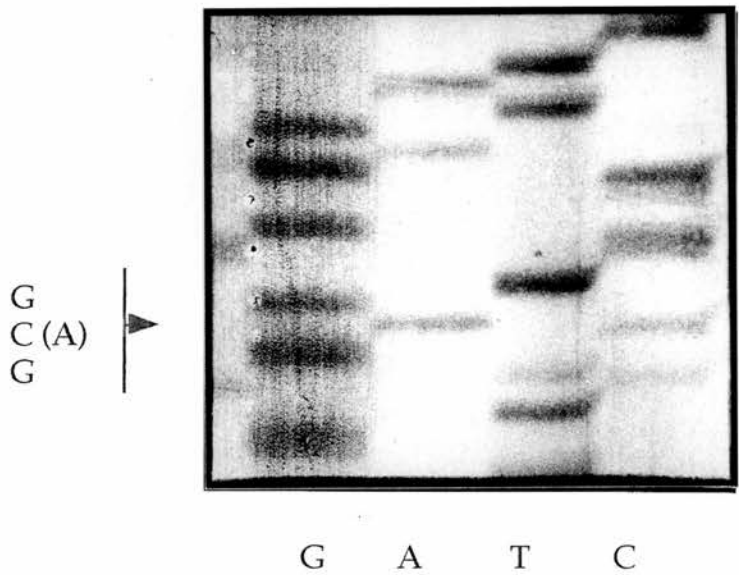
Mutations successfully characterised are demonstrated in Figure 4.4. They include the following 3 mutations from patients who had an abnormal SSCP pattern in their bladder washing samples:

Sample	Patient	Exon	Wild Type	Mutation	Codon
18	M	6	GAG(Glu)	GCG(Ala)	224
27	T	6	TAG	TAA	Upstream Splice Site
19	N	8	AGA(Arg)	ACA(Thr)	280

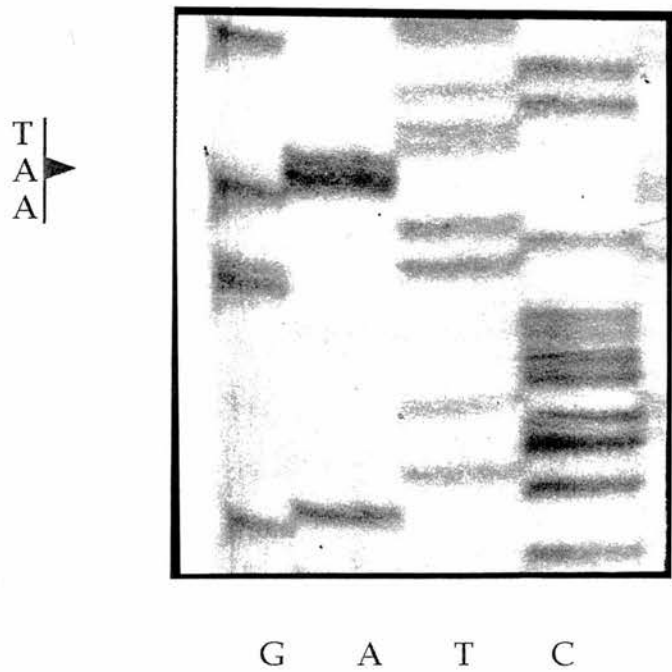
In addition the following 2 samples (from the same patient E) were sequenced in which the bladder washing had a normal SSCP pattern but in which the tumour sample was abnormal :

Sample	Exon	Wild Type	Mutation	Codon
9	8	GAC(Asp)	AAC(Asn)	281
24	8	GAC(Asp)	AAC(Asn)	281

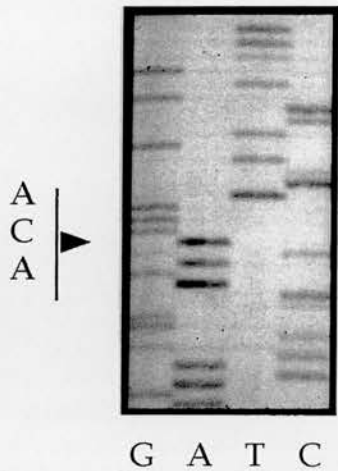
Figure 4.4
 Autoradiographs of sequencing gels confirming the presence of mutations in samples from 4 patients with bladder cancer.
 a) Bladder Washing Sample 18 from patient M demonstrating a GAG - GCG mutation in codon 224 (exon 6).



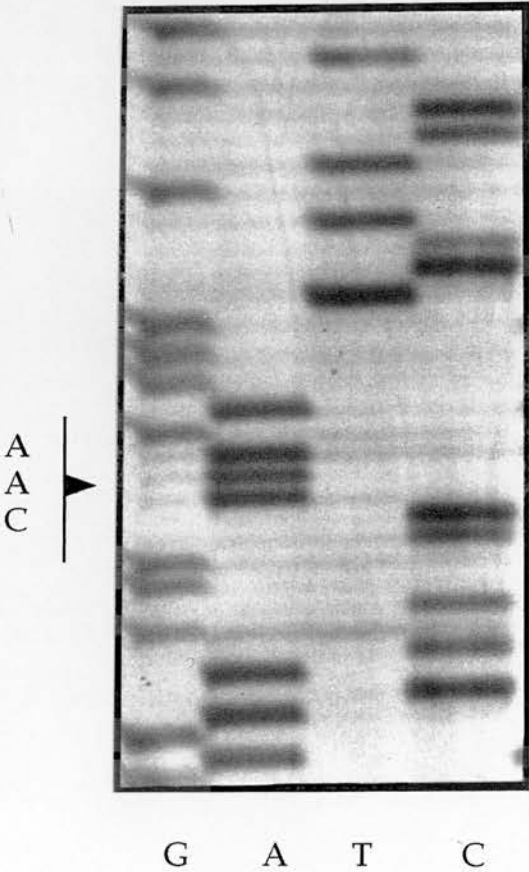
b) Bladder Washing Sample 27 from patient T demonstrating a TAG - TAA mutation at the 5' splice site of exon 6.



c) Bladder Tumour Sample 19 from patient N demonstrating a AGA - ACA mutation in codon 280 (exon 8). The bladder washing sample from this patient demonstrated the same SSCP abnormality as the Bladder Tumour Sample.



d) Bladder Tumour Sample 9 from patient E demonstrating a GAC - AAC mutation at codon 281 (exon 8). Bladder Tumour Sample 24 from the same patient demonstrated the same mutation on sequencing, but neither of the corresponding Bladder Washing Samples showed an SSCP abnormality.



The putative mutation in 3 specimens showing SSCP abnormalities in exon 7 could not be characterised. In all 3 cases when sequencing was attempted using amplified DNA from the washing sample and/or tumour the resulting sequence was indistinguishable from wild type. This was most likely due to the presence of a predominance of DNA of normal tissue origin in the sample. Subsequently the abnormally migrating bands seen on SSCP were excised from dried SSCP gels, the single stranded product eluted and used as the template for reamplification as a source of mutation carrying DNA for sequencing. However despite several attempts using bands cut from different gels it proved impossible to obtain a reamplification product for any of the samples tested for this exon; (this method had been successfully used for samples with mutations in other exons). It was felt likely that this was a methodological problem reflecting the nature of that particular pair of primers. Having failed to characterise the putative mutation in these 3 samples, the issue of whether or not the SSCP abnormalities seen actually reflected the presence of mutation in these samples merited consideration. In all 3 cases the same abnormal pattern was seen reproducibly on at least 4 occasions using the products of different PCR reactions. In addition for the 2 bladder washing samples concerned (samples 3 and 30) duplicate stored pellets were available. DNA was extracted from these and PCR - SSCP demonstrated the same abnormal migration pattern that had previously been seen. Furthermore although the originally studied fresh bladder tumour specimen from 2 of these cases failed to show the SSCP abnormality the paraffin embedded tissue did, the difference in pattern between samples most likely reflecting a genuine difference between them i.e. the presence of mutation carrying cells in variable amount rather than artifact. The observation that for

tumour sample 41 the corresponding washing appeared normal could be interpreted similarly.

4.5 SSCP Analysis of Tumour samples Without Corresponding Washing Samples.

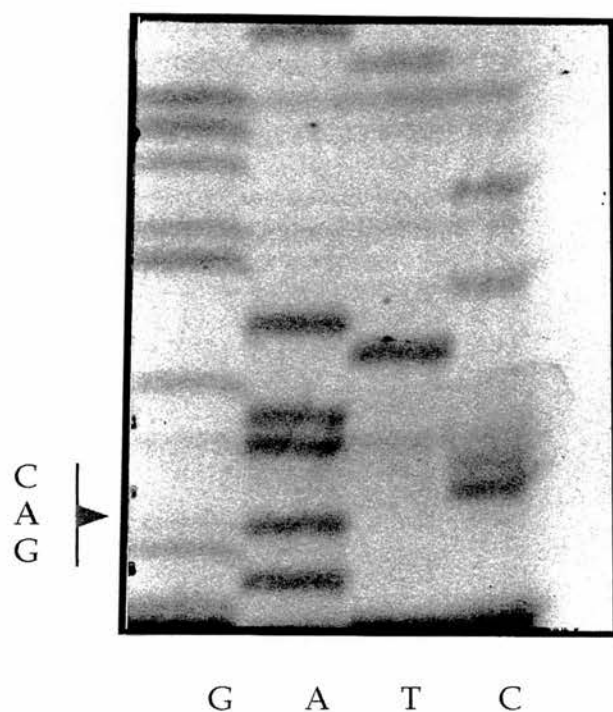
There were 2 tumour samples available for study for which there was no corresponding washing available. For completeness these were also screened for the presence of mutations in exon 5-8 of the p53 gene. Both were from patients with high grade transitional cell carcinomas of the bladder and 1 demonstrated an abnormality in exon 7 on SSCP with the abnormality seen under all 3 sets of gel conditions used. The mutation was sequenced as follows:

Exon	Wild Type	Mutation	Codon
7	CGG (Arg)	CAG(Gln)	248

This mutation is demonstrated in Figure 4.5. As discussed earlier sequencing exon 7 mutations by reamplifying abnormal single stranded DNA excised from dried gels did not prove technically possible. The high ratio of abnormal to normal sequence seen on the SSCP gel allowed this particular sample to be successfully sequenced direct from the PCR product without the need for band excision and reamplification.

Figure 4.5

Autoradiograph of a sequencing gel confirming the presence of a CGG - CAG mutation in codon 248 (exon 7) in a bladder tumour sample from a patient with a high grade transitional cell carcinoma of bladder from whom no bladder washing sample was collected.



4.6 Clinico-Pathological Characteristics of mutation Carrying Samples.

All mutation-carrying samples were from patients with high grade, poorly differentiated cancers. In each case the histology was of transitional cell carcinoma, although 2 samples from one patient also contained elements of squamous carcinoma, that is mixed histology (samples 9 and 24 from patient E). All but 1 of the mutation carrying samples were from patients with pT1 or pT2 tumours, invading either the lamina propria or deeper into detrusor muscle. The exception was a high grade pTa recurrence in a patient treated with intravesical chemotherapy for a pT1 tumour that had demonstrated the same SSCP abnormality (sample 26 from patient M).

Samples were available from a total of 8 patients with well or moderately differentiated bladder cancers (grade 1 or 2). No mutations were found in either the bladder washing or tumour samples from any of these patients.

Discussion

31 bladder washing samples from 27 patients with urothelial malignancy were screened for mutations in exons 5-8 of the p53 gene. Bladder washing samples from 5 patients with bladder cancer were found to contain evidence of the presence of p53 mutations. In each case it was possible to demonstrate SSCP abnormalities in the corresponding bladder tumour sample. It proved possible to characterise the mutation by direct sequencing in 3 of the 5 cases. In the other 2, both with abnormalities in the same exon, technical difficulties did not permit the successful reamplification of single stranded DNA bands excised from SSCP gels. However the reproducible finding of the same SSCP abnormalities in the bladder washings on repeat testing and the presence of the same abnormal pattern in tumour samples is highly indicative of the presence of mutation. Bladder tumour samples were also analysed in the same way. When the results of studying fresh tumour and paraffin embedded tissue are considered together a total of 9 out of 23 samples were shown to carry a mutation in the p53 gene, representing samples from 7 out of 20 patients studied. There were 4 instances therefore in which a mutation could be demonstrated in the tumour specimen but not in the corresponding bladder washing sample.

Several observations concerning the above results are worthy of note. Firstly, the detection of p53 mutations in tumour samples from 7 out of the 20 patients from whom paired tumour and washing samples were collected is consistent with previously published data, given that the cohort included 4 patients with moderately or well differentiated papillary superficial cancers that have a low incidence of mutations (Fujimoto et al 1992, Esrig et al 1993). Despite this there remains concern

that mutations were missed because of inadequate sampling of tumour. The fresh tumour samples taken at operation represent shavings of tumour resected endoscopically and were chosen macroscopically from the debris evacuated from the bladder at the completion of the operation. In retrospect it is quite likely that only a small proportion of tumour may have been present in some samples. This is borne out by the fact that in those instances in which the bladder washing showed a mutation but the fresh frozen tissue did not, the mutation could be demonstrated in DNA extracted from paraffin embedded tissue. As paraffin embedded tissue was only obtained for those samples in which the washing showed a mutation but fresh tumour did not, it remains possible other mutations went undetected. The proportion of tumour in the paraffin embedded samples was assessed by a pathologist (Dr. J. Webb, Western General Hospital) and was found to be variable and on some occasions very low (range <10% - 80%). It seems appropriate therefore to suggest that in any further studies of this nature on bladder cancer that DNA is extracted from microdissected tissue for analysis, or at least that the proportion of tumour in the sample is assessed prior to DNA extraction. Whether this tissue is frozen or paraffin embedded would depend on the proposed use of the DNA. Fresh tissue provides higher yields of longer fragments of well preserved DNA amenable to study with a larger range of techniques but paraffin embedded tissue is often more readily available, easier to store and yields DNA of adequate quality for a number of PCR based techniques.

Five bladder washing samples from different patients were found to contain mutations. For 1 patient 2 samples taken on different occasions were studied and of these only 1 was positive for the presence of a

mutation and the other negative. In addition primary tumour from 2 patients showed a mutation when the bladder washing appeared normal (in 1 case on 2 separate occasions). In total there were 9 washing samples available from patients whose primary tumour harboured a mutation, with the mutation detected in the washing in 5 out of 9 cases (56%). It can therefore be seen that by analysing the bladder washing samples alone there were a number of false negative results, and that the proportion of false negatives may well be an underestimate in view of the potential sampling problem with tumour tissue discussed above. As bladder washing samples were only taken when there was obvious tumour visualised at cystoscopy, it seems likely that the washing sample would have contained at least a small proportion of tumour cells and therefore that the most likely explanation for the false negative results was the limited sensitivity of SSCP in detecting minority cells in a mixed cell population. In this study no attempt was made to quantify the proportion of malignant cells in the bladder washings by conventional cytological techniques, mainly because of the inherent unreliability of such techniques (Vet et al 1996). However the proportion would be expected to vary depending on the size of the tumour, its biological properties in particular the degree of cellular adhesion and other factors such as the amount of ulceration and necrosis on the tumour's surface (the part of the tumour sampled by bladder washing).

The original study on p53 mutations in bladder cancer reported by Sidransky et al (1991) as well as characterising mutations in a cohort of 16 bladder cancers used mutation specific oligonucleotide probes to study the corresponding urine samples from 3 patients with mutation carrying tumours. Each of these 3 samples contained the same mutation as the

tumour but such mutation-carrying cells were estimated to represent only 1 -7 % of the cellular component of the urinary sediment. It is thus apparent that the proportion of tumour derived cells in that one small study overlaps with the limits of sensitivity of SSCP that was demonstrated in the cell mixing experiments of the current study described in Chapter 3. It however is important to note that in at least some patients in the current study the proportion of tumour cells in the bladder washing samples would appear to be very much greater . This is demonstrated by the prominent relative intensity of the abnormally migrating SSCP band compared to the normal bands, as visualised on autoradiographs, and is consistent with findings of other investigators (Mao et al 1996, Xu et al 1996). This may in part be due the increased cellularity of bladder washings compared to voided urine samples. In addition it has been suggested that details of sample storage may have a bearing on proportion of malignant cells in a sample, with the possibility that malignant cells may have a survival advantage in certain conditions (Mao et al 1996).

In the above studies a total of 8 different mutations were identified in one or more of the sample types studied. It is noted that only 2 of the mutations were unambiguously identifiable under all 3 sets of gel conditions employed for that particular exon, and 2 mutations were only identified under 1 set of conditions. The importance of using at least 2 sets of SSCP gel conditions to maximise mutation detection is thus apparent.

It is relevant to discuss the above results in the light of previous related work. It has been demonstrated previously that cancer related genetic abnormalities can be detected in urine and bladder washing samples de

novo, using H-ras as a marker (Levesque et al 1993, Fitzgerald et al 1995). However H-ras mutations may be primarily important in the more indolent, less aggressive types of bladder cancer (Fitzgerald et al 1995) and at other sites can occur in benign proliferative disorders (Hollywood et al 1995). The findings of the current study, along with 2 reports published whilst this study was in progress confirm that p53 mutations (an event relevant to more clinically aggressive bladder cancers) can be detected in such samples using techniques that do not require prior characterisation of the mutation being sought (Vet et al 1996, Xu et al 1996). These are therefore novel findings as the only prior reports on p53 and urine samples had utilised mutation specific oligonucleotides (Sidransky et al 1991, Hruban et al 1994). The current work is the first that correlates the p53 mutational status of bladder washings with corresponding tumour specimens, although Xu et al (1996) addressed this important issue using direct sequencing of DNA extracted from urinary sediments. Differences in results obtained bear further discussion. It is apparent from the current study that analysing bladder washing samples for p53 status has an appreciable false negative rate, that is sensitivity is a limiting factor and reasons for this are discussed earlier. There are few data comparing the sensitivity of SSCP and direct sequencing in a mixed cell population, though it has been reported that SSCP is possibly more sensitive in this regard (Smith et al 1992, Wu et al 1993). It is of interest therefore that 24 of 30 urine samples from 8 patients with bladder cancers harbouring a p53 mutation demonstrated the relevant mutation by direct sequencing (Xu et al 1996). Furthermore the 6 remaining cases coincided with negative cystoscopies. These data suggest enhanced sensitivity compared to the current study. Of additional interest is the observation that abnormal p53 can be detected in bladder washings or urine samples by SSCP and direct

sequencing respectively despite normal cystoscopic appearances in patients with a prior history of bladder cancer (Vet et al 1996, Xu et al 1996). That these findings were achieved using mutation detection techniques of limited sensitivity in a mixed cell population emphasises that the detection of molecular abnormalities may offer earlier detection of relapsed or recurrent disease than is possible cystoscopically.

The results therefore demonstrate that it is possible to detect tumour suppressor gene abnormalities in bladder washings from patients with bladder cancer, using the p53 gene as a marker. Such abnormalities can be confirmed and characterised by direct sequencing and the presence of the same mutation in corresponding tumour samples has been confirmed. Thus the primary aims of the pilot study were fulfilled. Being a pilot study a major objective is to enable the rational design of further studies. Such further studies would fall naturally into 3 groups with different objectives:

- 1) From the results of this study and the results of others it is apparent that the detection de novo of oncogene abnormalities in urine and bladder washing samples is achievable albeit with potentially limited sensitivities. Such samples are an ideal way to study the entire urothelium of the bladder and as such hold great promise for study of the early genetic events in groups of subjects at high risk of developing bladder cancer and of progressing from superficial to invasive disease. Such studies would ideally involve the collection of serial samples prospectively and subsequent analysis in the light of clinical progression. Continuing advances in the technologies of molecular biology may overcome some of the problems of sensitivity, as may the use of cell sorting techniques utilising cytokeratin labelling to enrich the epithelial

component of samples (Griffiths et al 1995). There is potential therefore for such approaches to add significantly to the understanding the molecular biology of the natural history of bladder cancer.

2) The second potential area for further study is the utilisation of molecular abnormality detection in the screening of asymptomatic subjects for the detection of clinically important bladder pathologies early in their natural history of the disease when medical intervention is likely to have a beneficial effect on outcome. In this regard work on microsatellite analysis is potentially interesting (Mao et al 1996). However to be introduced into clinical practice such a test or series of tests would have to meet stringent standards in terms of sensitivity (to avoid inappropriate reassurance in the presence of significant disease) and specificity (to avoid unnecessary investigation to pursue the diagnosis in the presence of a false positive test). Other issues such as subject convenience, aspects of psychological and physical distress to subjects, cost effectiveness and resource priorities would need to be addressed. Thus whilst intellectually and clinically appealing major developments are awaited before a clinically robust screening assay for the early detection of bladder cancer is realised.

3) The final avenue of potential further research that merits discussion is the use of molecular markers to aid follow up of patients with bladder cancer who have undergone bladder preserving treatments, and whom traditionally have been followed up primarily with regular cystoscopic examination. With the advent of automated microchip sequencing techniques allowing rapid characterisation of genes of interest (Kozal et al 1996) it may soon be practicable to screen tumour specimens routinely for abnormalities in a variety of genes. With further elucidation of the molecular biology of bladder cancer, genes to study could be chosen that

maximised the chances of finding an abnormality for an individual patient taking into account for instance factors such as histology, stage and grade. Having identified a mutation in an individual patient's tumour a sensitive mutation specific assay could be used to determine the presence or absence of that mutation in urine or bladder washing samples following the completion of treatment, and thus inferring the presence or absence of disease. Such an approach would obviously require rigorous standards of quality control given the potential for contamination in PCR based techniques. Prospective evaluation of its clinical sensitivity in particular with regards to correlation to cystoscopic findings would be imperative including evaluation of the significance of a positive molecular test in the absence of cystoscopic abnormality as such findings have already been described (Mao et al 1996, Xu et al 1996). It is possible that sensitive molecular techniques may reduce the need for cystoscopy, permitting cystoscopy only when a positive test suggests the likely presence of tumour. At first glance the level of scientific effort required per patient in terms of both cost and time may appear prohibitive, however with increasing automation and speed of molecular biological techniques unit costs may fall, and such assays may eventually become readily achievable in hospital clinical laboratories. When compared to the expense and patient inconvenience of regular cystoscopies, including the inherent risks of general anaesthesia in a predominantly elderly patient population with their associated smoking related comorbidities, pursuing the development of assays utilising molecular abnormalities as biochemical markers of malignancy would appear a worthwhile avenue for further studies.

CHAPTER 5

STUDIES ON p53 MUTATIONS IN NIPPLE ASPIRATE FLUIDS FROM PATIENTS WITH BREAST CANCER

Aims

The objective of this part of the study was to determine whether it was possible to detect the presence of malignancy in the breast fluid (obtained by nipple aspiration) of patients with breast cancer by using mutations in the p53 gene as a biochemical marker. This was a pilot study, building on previous observations that it was possible to amplify DNA for genes such as c-erbB-2, p53 and aromatase from nipple aspirate fluid. It was clear from the published literature that the quantity of fluid obtained for study may be small. Therefore the plan of investigation was different to that employed for the bladder cancer arm of the study described in Chapter 4. It was decided first to screen the breast tumour samples for the presence of mutations in exons 5-8 of the p53 gene and then investigate whether those mutations found could be demonstrated in the corresponding nipple aspirate sample. This approach minimises the amount of fluid sample required to test the hypothesis, as potentially only 1 PCR reaction is required per aspirate sample rather than 4 if the nipple aspirate fluid was to be screened for mutations in all 4 exons of interest, assuming that a mutation was demonstrated in only 1 exon per tumour.

Results

5.1 Nipple Aspirate Collection

Breast fluid collection by nipple aspiration was attempted in 30 subjects and fluid obtained from 21 (70%). The clinical details of the subjects from whom a nipple aspirate sample was obtained are tabulated in Appendix 3,

and include: the patient's age, the size, histological type, grade and oestrogen receptor status of the tumour, the nature of the operation performed and finally whether or not a sample of macroscopically normal breast tissue was obtained.

5.2 Screening of Breast Tumour Specimens for Mutations in Exons 5-8 of the p53 Gene.

Paired samples of breast tumour and nipple aspirate fluid from the same breast were obtained from 21 patients with histologically confirmed breast cancer. One patient who underwent a mastectomy was demonstrated preoperatively to have 3 discrete masses, both clinically and mammographically; tumour samples were collected from each of the 3 lesions, giving a total of 23 tumours from 21 subjects. DNA was extracted from either fresh frozen or paraffin embedded tumour tissue. PCR-SSCP was performed for each of the 4 exons of interest in turn, using 3 sets of SSCP conditions for each exon as discussed in Chapter 3. For each exon studied the PCR reaction included a negative, no template control to demonstrate absence of contamination of the PCR reaction and a sample both of wild type and mutation carrying cell line DNA for that exon; the wild type sample acting as a normal reference control, to which the migration pattern of the tumour DNA samples could be compared and the mutation carrying sample allowing comparison of migration pattern to that of previous gels run under the same conditions ensuring interassay consistency. When the migration pattern of a sample indicated the possibility of a p53 mutation a further PCR reaction was performed and SSCP repeated. If there was a disparity between the 2 results the PCR-SSCP was repeated again and the sample only considered to

potentially contain a mutation if the same abnormal migration pattern was seen on at least 2 occasions, the abnormal pattern otherwise being attributed to artifact.

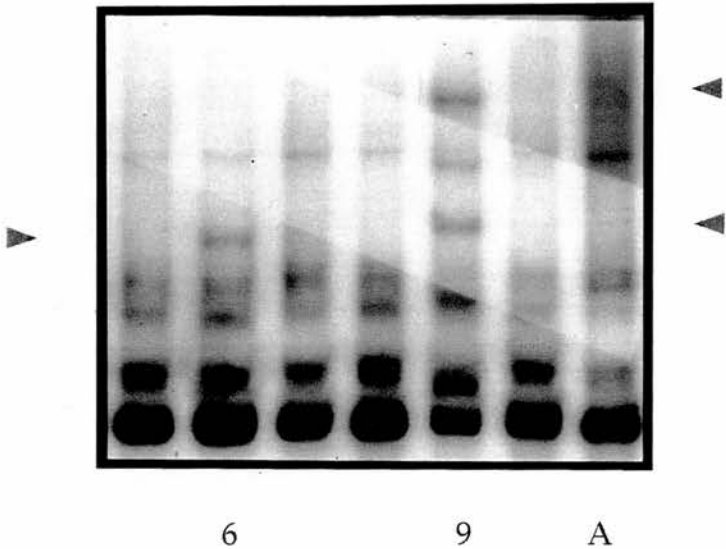
A total of 4 samples demonstrated an abnormal SSCP pattern, 2 in exon 5, 1 in exon 6 and 1 in exon 8. These abnormalities are demonstrated in Figure 5.1, and the results are summarised in Table 5.1 (exons 5 and 6) and Table 5.2 (exons 7 and 8). It can be seen that whilst 3 out of 4 of the putative mutations were seen on all 3 sets of gel conditions, the remaining 1 (sample 6) was seen on only one set of conditions.

5.3 Screening of Normal Breast Tissue Specimens from Tumour Bearing Breasts for mutations in exons 5-8 of the p53 gene.

Macroscopically normal breast tissue was available from the tumour-bearing breast in 5 out of the 21 patients studied. Exons 5-8 of the p53 gene were screened for mutations using PCR-SSCP as described for the breast tumour samples. No mutations were demonstrated in any of the 5 samples. These 5 samples included 2 from patients whose tumours displayed an abnormal SSCP pattern for 1 of the exons studied (samples 6 and 18).

Figure 5.1 Autoradiographs of SSCP gels demonstrating the presence of abnormal SSCP migration patterns suggesting the likely presence of mutation in breast cancer tumour samples 1,6,9 and 18.

a) Exon 5. An abnormal extra band is seen in sample 6 and 2 abnormal extra bands are seen in sample 9 (indicated by arrows); lane A is an A2780 cell line normal control.



b) Exon 6. An abnormal extra band is seen in sample 1 (indicated by arrow); lane M is a T47D cell line known mutant control and lane A is an A2780 cell line normal control.

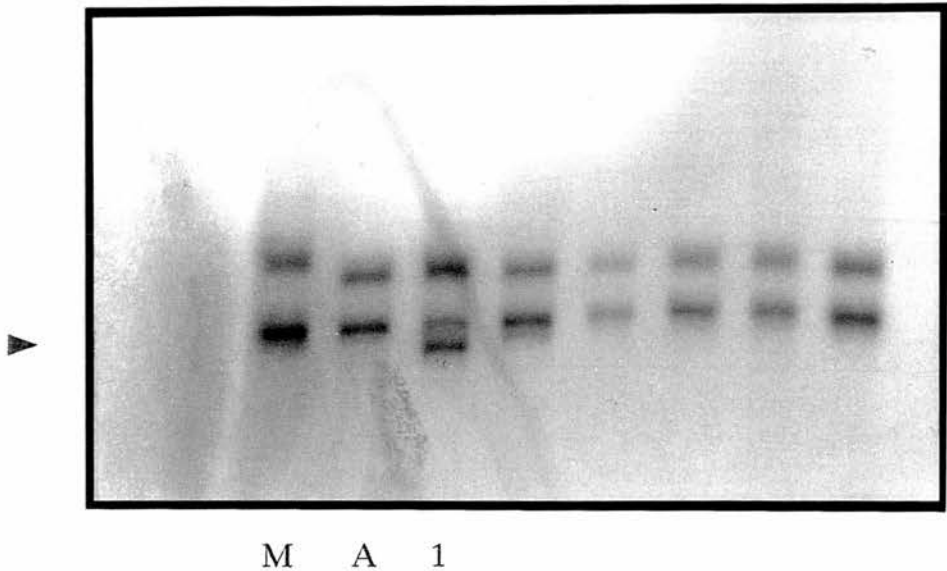


Figure 5.1 contd

c) Exon 8. Abnormal extra bands are seen in sample 18. SS and DS indicate the normal migrating single and double strands respectively. In addition 2 extra single strand bands are seen (indicated by arrows) as well as an extra double stranded band (indicated by *). These appearances indicate the presence of a mutation carrying a sizeable deletion giving rise to PCR products that migrate faster and therefore further than their wild type counterparts. Lanes labelled A carry A2780 cell line normal controls.

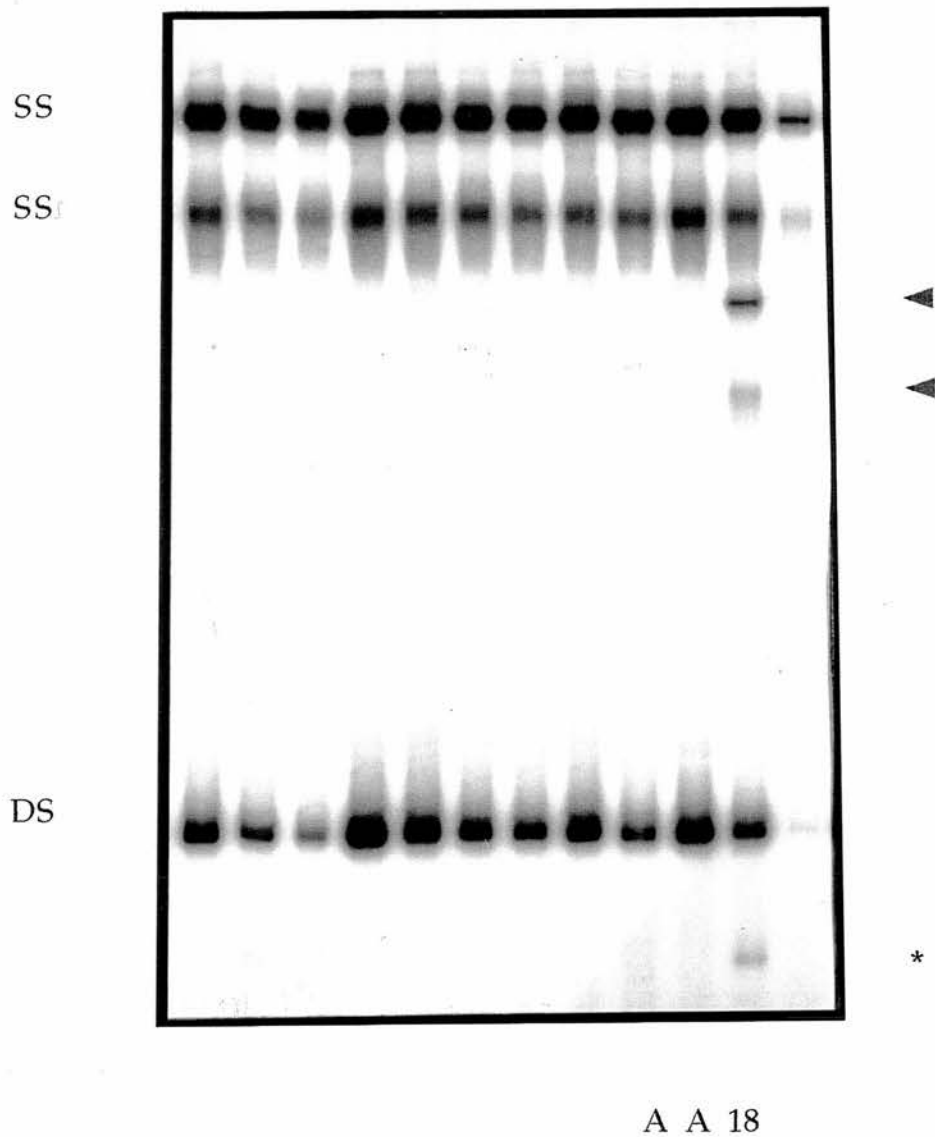


Table 5.1
Results of SSCP analysis of Exons 5 and 6 of the p53 gene in 23 Breast Tumour Samples

		Exon 5	Exon 5	Exon 5	Exon 6	Exon 6	Exon 6
Patient	Sample	Gel A	Gel C	Gel D	Gel A	Gel C	Gel D
A	1	N	N	N	ABN	ABN	ABN
B	2	N	N	N	N	N	N
B	3	N	N	N	N	N	N
B	4	N	N	N	N	N	N
C	5	N	N	N	N	N	N
D	6	N	N	ABN	N	N	N
E	8	N	N	N	N	N	N
F	9	ABN	ABN	ABN	N	N	N
G	10	N	N	N	N	N	N
H	12	N	N	N	N	N	N
I	13	N	N	N	N	N	N
J	14	N	N	N	N	N	N
K	15	N	N	N	N	N	N
L	16	N	N	N	N	N	N
M	17	N	N	N	N	N	N
N	18	N	N	N	N	N	N
O	20	N	N	N	N	N	N
P	21	N	N	N	N	N	N
Q	23	N	N	N	N	N	N
R	25	N	N	N	N	N	N
S	26	N	N	N	N	N	N
T	27	N	N	N	N	N	N
U	28	N	N	N	N	N	N

N : Normal SSCP Pattern

ABN: Abnormal SSCP Pattern

Gel A: 10% acrylamide, 1.3% bisacrylamide, no glycerol, 40°C

Gel C: 8% acrylamide, 2% bisacrylamide, 10% glycerol, 40°C

Gel D: 10% acrylamide, 1.3% bisacrylamide, 10% glycerol,
room temperature

Table 5.2
Results of SSCP analysis of Exons 7 and 8 of the p53 gene in 23 Breast Tumour Samples

		Exon 7	Exon 7	Exon 7	Exon 8	Exon 8	Exon 8
Patient	Sample	Gel B	Gel C	Gel D	Gel B	Gel C	Gel D
A	1	N	N	N	N	N	N
B	2	N	N	N	N	N	N
B	3	N	N	N	N	N	N
B	4	N	N	N	N	N	N
C	5	N	N	N	N	N	N
D	6	N	N	N	N	N	N
E	8	N	N	N	N	N	N
F	9	N	N	N	N	N	N
G	10	N	N	N	N	N	N
H	12	N	N	N	N	N	N
I	13	N	N	N	N	N	N
J	14	N	N	N	N	N	N
K	15	N	N	N	N	N	N
L	16	N	N	N	N	N	N
M	17	N	N	N	N	N	N
N	18	N	N	N	ABN	ABN	ABN
O	20	N	N	N	N	N	N
P	21	N	N	N	N	N	N
Q	23	N	N	N	N	N	N
R	25	N	N	N	N	N	N
S	26	N	N	N	N	N	N
T	27	N	N	N	N	N	N
U	28	N	N	N	N	N	N

N : Normal SSCP Pattern ABN: Abnormal SSCP Pattern

Gel B: 8% acrylamide, 2% bisacrylamide, no glycerol, 40C
Gel C: 8% acrylamide, 2% bisacrylamide, 10% glycerol, 40C
Gel D: 10% acrylamide, 1.3% bisacrylamide, 10% glycerol,
 room temperature

5.4 Characterisation of p53 Mutations by Direct DNA Sequencing.

The 4 samples with abnormal SSCP pattern were subjected to direct sequencing of the putative mutation carrying exon. For samples 1, 6 and 9 template DNA for sequencing purposes was obtained either by reamplification of single stranded DNA eluted from SSCP gels or direct from a non-radiolabelled double stranded PCR product. Sample 18 (Figure 5.1c) demonstrated not only abnormally-migrating single strands on SSCP but also an abnormal double stranded product which migrated further than the wild type product on the SSCP gel implying the presence of a sizeable deletion. To sequence this sample the PCR reaction (non radiolabelled) was repeated and the products run on a 1.5% agarose gel. The presence of 2 double stranded products was confirmed and the abnormally migrating band was excised. DNA was extracted from the gel, reamplified and then sequenced.

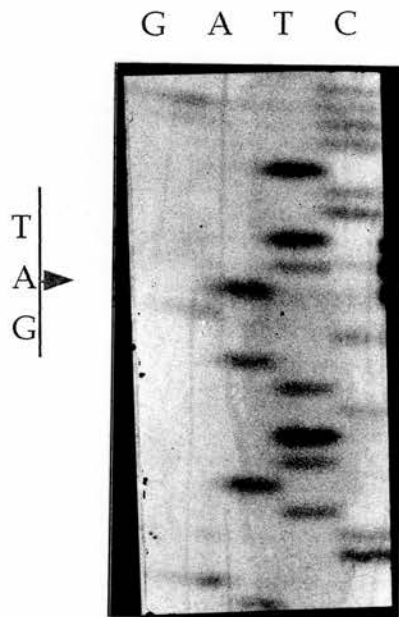
In each of the 4 cases the presence of a mutation was confirmed (Figure 5.2) and the details are tabulated in Table 5.3.

Table 5.3

Sample	Exon	Wild Type	Mutation	Codon
1	6	CAG (Gln)	TAG(stop)	192
6	5	CAT (Pro)	CGT (Arg)	179
9	5	GGT	GTT	(3') downstream splice site
18	8		48 base pair in frame deletion of codons 275-290 inclusive.	

Figure 5.2. Autoradiographs confirming by direct sequencing the presence of p53 mutations in breast cancer tumour samples 1,6,9 and 18.

a) Sample 1; CAG - TAG mutation at codon 192 in exon 6.



b) Sample 6; CAT - CGT mutation at codon 179 in exon 5.

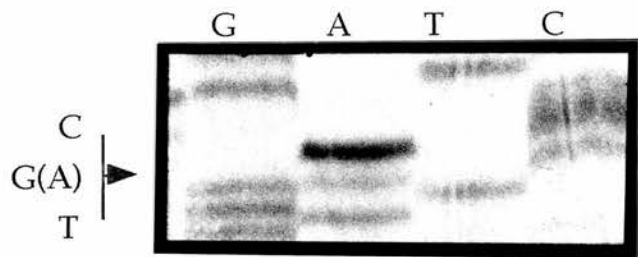
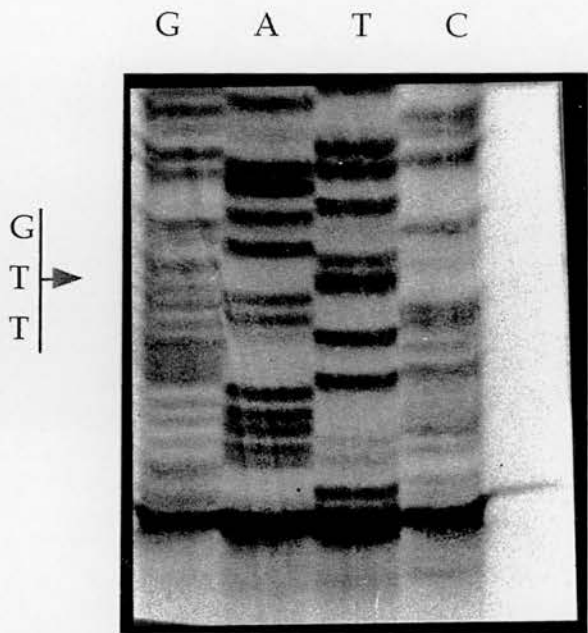
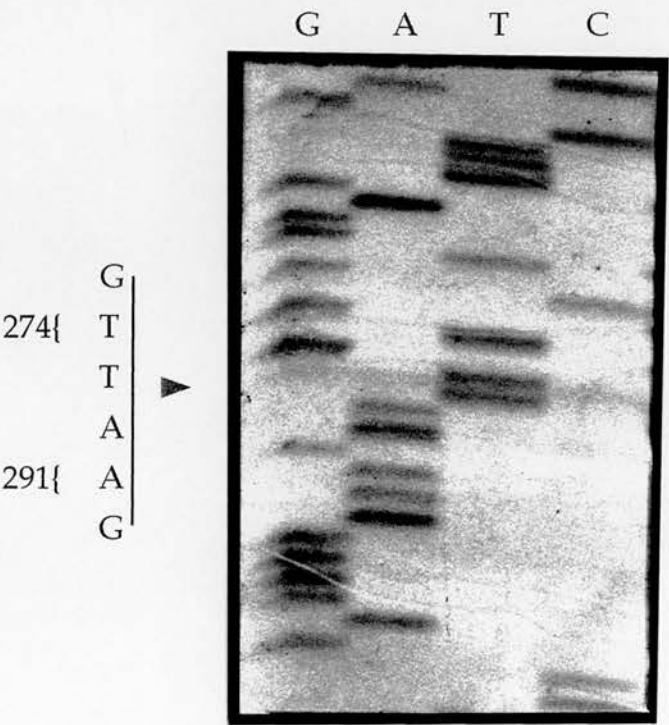


Figure 5.2 contd.

c) Sample 9; GGT - GTT mutation at 3' splice site of exon 5.



d) Sample 18; 48 base pair in-frame deletion of codons 275-290 of exon 8.



5.5 PCR Studies on Nipple Aspirate Samples

Following the above experiments characterising the mutations present in breast tumours, the nipple aspirate samples from those subjects whose tumour carried a mutation were put aside for later analysis. The remaining fluid samples (that is those from subjects whose tumour was not found to carry a p53 mutation) were used in studies attempting to define an effective method to prepare the DNA in the samples for PCR.

Two methods were used:

1) The first method involved the addition of a sample of nipple aspirate fluid to a tube prior to the addition of the other reagents for a PCR reaction as described by Motomura et al (1994). Duplicate samples (5-10 μ l) of nipple aspirate fluid from 1 subject were used as PCR template in 100 μ l PCR reactions. No PCR product was obtained in either case. Aliquots (10 μ l) of the first round amplification were used as substrate for a second reaction yielding a product in 1 case. A second experiment using duplicate aliquots of nipple fluid from a further subject yielded no product for either sample on either first or second rounds of amplification.

2) The second method involved the addition of a lysis buffer to the nipple aspirate fluid and subsequent incubation prior to the addition of the remaining PCR reagents and thermal cycling. On the first occasion this was attempted 2 NAF samples were split between 4 PCR tubes, but no product was seen from any or the positive control indicating the whole reaction had failed and had to be disregarded. In a further experiment a large sample was split between 3 tubes, lysed then amplified. No product

was seen from any of the 3 samples but the positive control on this occasion yielded a product. A further round of amplification using product from the first reaction as template for the second again yielded no product.

It can be seen that using the above 2 methods PCR product was seen from only 1 of 7 reactions and in that 1 case this was only following reamplification in a second PCR. There were 3 main possibilities that could account for this: firstly there was little or no DNA in the samples tested; secondly, that despite using 2 methods previously reported as having been used successfully to amplify the DNA from small samples, the DNA present was not being released into solution; and thirdly that the nipple fluid sample contained a factor inhibiting what had been, in the investigator's hands, robust PCR conditions. It was felt most appropriate to investigate the latter possibility, as if that suspicion was confirmed a DNA extraction method involving separation of the resultant DNA from the sample fluid would be mandatory.

5.6 Testing The Hypothesis That Nipple Aspirate Fluid Contains A Factor Inhibiting PCR Reactions

Nipple aspirate fluid was added to a known quantity of A2780 cell line DNA prior to PCR. The intensity of the product was compared to that of the same quantity of A2780 DNA amplified on the same reaction run. All reactions amplified exon 5 of the p53 gene.

1) In the first experiment 1 μ l (100ng) of A2780 DNA was amplified in the

presence and absence of 5 μ l of nipple aspirate fluid. The PCR run included a no template negative control and a tube containing 5 μ l of nipple aspirate fluid but no cell line DNA. The nipple aspirate fluid alone and the negative control yielded no product on agarose gel electrophoresis and the intensity of product from amplification of the the mixed A2780 DNA and aspirate was less than that of the A2780 alone (Figure 5.3a). The apparent partial inhibition observed may have simply been accounted for by inter tube variations in amplification.

2) To address this issue a second experiment was performed in an identical manner to experiment 1, save that 2 tubes rather than 1 containing A2780 template alone were set up. On this occasion, whilst both the A2780 samples revealed products of similar intensity the A2780/ aspirate mix showed no visible product. The aspirate alone sample and negative controls again yielded no product (data not shown).

3) The final experiment utilised 3 nipple aspirate samples. Approximately 5 μ l of each sample was added to separate tubes each containing 1 μ l (100ng) of A2780 DNA. A negative no template control and 2 x A2780 only tubes were also set up. There was insufficient aspirate fluid for aspirate alone tubes to be set up. Two of the 3 A2780/ aspirate mix samples yielded no product as did the no template control and the remaining A2780/ aspirate mix sample had a product intensity less than either of the 2 A2780-only specimens (Figure 5.3b).

Figure 5.3

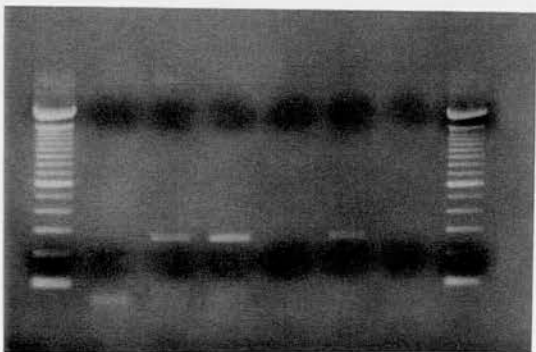
Agarose gel electrophoresis demonstrating the effect of mixing nipple aspirate fluid and cell line DNA on PCR product yield.

a) Lane 1: DNA ladder. Lane 2: no-template negative control. Lane 3: 5µl NAF as PCR substrate. Lane 4: 100ng A2780 cell line DNA as PCR substrate. Lane 5: mix of 5µl NAF and 100ng A2780 as PCR substrate. No PCR product is visualised when NAF alone is the substrate and the yield of product appears less for the mix of cell line DNA and NAF, than for the cell line DNA alone.



1 2 3 4 5

b) Lanes 1 and 8: DNA ladder. Lane 2: no-template negative control. Lanes 3 and 4: 100ng A2780 cell line DNA as PCR substrate. Lane 5 - 7: mix of 5µl NAF from 3 different subjects and 100ng A2780 as PCR substrate. The additon of NAF to cell line DNA results in absence of product in lanes 5 and 7 and decrease in product intensity in lane 6, implying an inhibitory effect (noting however that the product intensity differs between the 2 A2780 samples).



1 2 3 4 5 6 7 8

Taken together the 3 experiments support the presence in nipple aspirate fluid of agents which either partially or completely inhibit the PCR reaction. Inhibition was complete on 3 out of 5 occasions. It was therefore decided to attempt DNA extraction using the Qiagen Tissue kit employing a modification of a method described by the manufacturers for extracting DNA from small quantities of body fluids dried on microscope slides (Chapter 2.4.1e). Five extractions were performed using fluid samples from 4 patients and a PCR product obtained after 1 round of amplification in 2 case and after a 2nd round in another. A decision was taken to proceed with this method for the index cases.

5.7 SSCP analysis of nipple aspirate samples from subjects whose breast cancer revealed a p53 mutation.

DNA was extracted from the nipple aspirate samples of the 4 subjects whose breast tumours were shown to contain a p53 mutation. PCR-SSCP was performed for each sample only for the exon previously shown in the corresponding tumour sample to carry a mutation. Some previous reports on PCR using small samples have utilised either an initial PCR reaction of a conventional number of cycles and then used an aliquot of the first round product as the DNA template for a second round of amplification (Kovach et al 1991) or used upwards of 50 cycles of amplification replenishing the DNA polymerase mid procedure (to ensure continued activity) (Frye et al 1989); the aim of both procedures being to maximise amplification. In the current study the following approach was adopted. A first round of amplification was performed in a 100 μ l PCR reaction (non radiolabelled) using 2 different quantities of

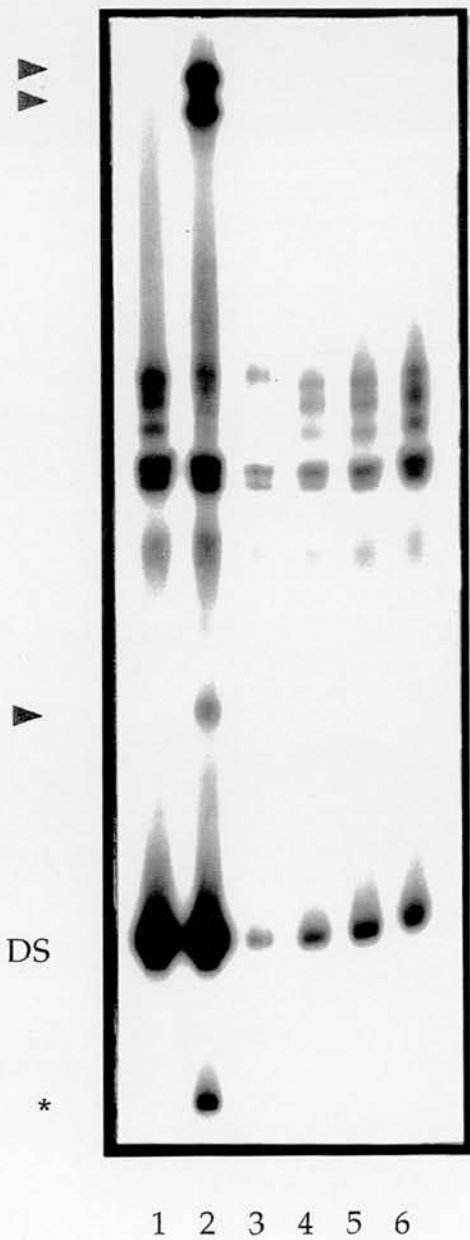
sample as template (2µl or 10µl). Agarose gel electrophoresis failed to demonstrate a clear DNA product for any of the 4 samples. Then a 20µl ³²P labelled PCR was performed using the following as template:

- 1) 2µl DNA extracted from aspirate sample
- 2) 10µl DNA extracted from aspirate sample
- 3) 2µl aliquot of first round product from the initial 100µl PCR in which 10µl of DNA template was used
- 4) 10µl aliquot of first round product from the initial 100µl PCR in which 10µl of DNA template was used

Appropriate positive and negative controls were included in the reaction as were samples of DNA from the corresponding mutation carrying tumours. SSCP analysis was performed, as previously, on 3 sets of gel conditions for each exon studied. Nipple aspirate samples corresponding to tumour samples 6 and 9 yielded no product despite prolonged autoradiography for any of the 4 aspirate templates used. Three and 4 of the templates from the aspirates corresponding to tumour samples 1 and 18 respectively yielded product visualised on SSCP gels. No abnormally migrating bands were demonstrated (Figure 5.4), that is using SSCP, no evidence of tumour derived genetic material could be demonstrated in the 4 aspirate samples studied.

Figure 5.4

SSCP analysis of nipple aspirate fluid sample corresponding to tumour sample 18. Lane 1 is an A2780 cell line normal control. Lane 2 is tumour sample 18 demonstrating abnormal single strands (indicated by arrows) and an abnormal double stranded product (labelled *; DS representing the normal double stranded product). Lanes 3 - 6 represent nipple aspirate samples from the same patient; it can be seen that no abnormal bands are visualised.



5.8 Mutation Specific Amplification of Sample 18

As described earlier breast tumour sample 18 was demonstrated to carry a sizeable deletion. A pair of mutation specific primers were designed to amplify this mutation.

The primers were as follows:

sense	5'	GC TTT GAG GTG CGT GTT AAG	3'
corresponding codon		269 270 271 272 273 274 291	

antisense	5'	CAGTGCTAGGAAAGAGGCAA	3'
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The antisense primer was entirely intronic with its 5' end being 98 bases downstream from the 3' end of exon 8. The sense primer spanned the deletion and was therefore mutation specific.

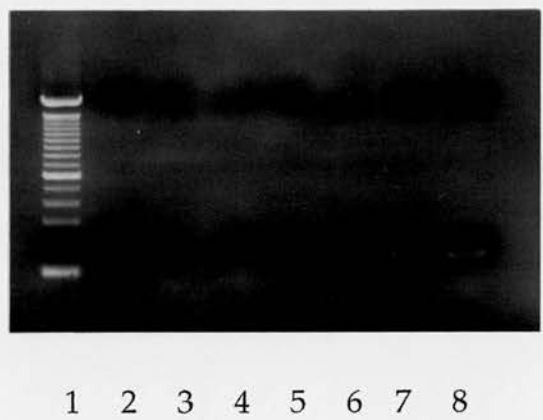
Following optimisation experiments it was found that this pair of primers would specifically and reproducibly amplify the mutation, if an annealing temperature of 55°C was used with a MgCl₂ concentration of 1mM , all other reaction conditions being as described in Chapter 2.

5.9 Mutation Specific Amplification of Nipple Aspirate Corresponding To Tumour Sample 18.

Having failed by SSCP to detect the presence of tumour derived DNA in the aspirate corresponding to tumour sample 18, the possibility was investigated that the use of mutation specific primers may demonstrate its presence. The rational was that mutation specific amplification allows amplification of as little as 1 copy in 10^{5-6} (mutant: wild type ratio) (Cha et al 1992, Jacobson and Mills 1994, Rhodes et al 1997) a sensitivity several fold better than established in this study for SSCP. Using the PCR reaction conditions described in the previous section a 100 μ l PCR reaction was performed, using as template 2 μ l and 10 μ l aliquots of aspirate DNA extract, as well as wild type DNA (A2780 cell line) and tumour sample 18 DNA along with a negative, no template control. Sample 18 tumour DNA was the only reaction to yield a product that could be visualised on agarose gel electrophoresis (Figure 5.5). A further 10 μ l aspirate DNA sample was subjected to PCR and again yielded no product. Aliquots of the first round of PCR reactions were used as template for second round amplification and no definite bands were observed on agarose gel electrophoresis. It can be concluded that despite the use of mutation specific primers it was still not possible to demonstrate the presence of tumour derived mutation carrying DNA in the nipple aspirate fluid of this subject.

Figure 5.5

Agarose gel electrophoresis of a PCR using primers specific for the mutation in tumour sample 18. Lane 1 contains a 100 base pair DNA ladder, lane 2 a no-template control, lanes 3 and 4 A2780 cell line DNA (as a non-mutation carrying negative control), lanes 5 and 6 sample 18 aspirate DNA and lanes 7 and 8 sample 18 tumour DNA. A PCR product is visualised only in lanes 7 and 8 (sample 18 tumour DNA) and not in the corresponding nipple aspirate samples.



Discussion

Employing standard methodologies it was possible to demonstrate the presence of p53 gene mutations in breast tumours from 4 out of 21 patients (19%), and characterise those mutations found by direct sequencing. This proportion of mutation carrying tumours whilst consistent with previous data is at the lower end of the range of reported frequencies (Andersen et al 1993, Dunn et al 1993, Merlo et al 1993, Sasa et al 1993, Tsuda et al 1993, Saitoh et al 1994). There are several possible explanations for this: i) the presence of normal tissue may mask tumour mutation by dilution of abnormal DNA, ii) a lack of sensitivity of SSCP in detecting the presence of mutations, iii) a statistical phenomenon of a small series, iv) the fact that only exons 5-8 were studied or v) bias in terms of recruitment. Each of these factors will be considered in turn. The tumour samples were macroscopically dissected from visible tumour masses by experienced pathologists and it is unlikely that the samples contained a lesser proportion of tumour than could be detected by SSCP (1-30% as demonstrated in Chapter 3). SSCP has been demonstrated to detect upwards of 70-90% of mutations present (Grompe 1993, Ravnik-Glavac et al 1994) and given the conditions employed in this study including the use of 3 gel conditions for each sample it seems unlikely that a large proportion of mutation carrying tumours were missed. The sample size was small and a statistical quirk may account for the low proportion of mutation carrying tumours found. As described in the introduction many of the series reporting on p53 mutations screened only the highly conserved regions of the gene (as in this study). Whilst mutations are described in other regions they are less common; nevertheless the restriction of mutation screening to exons 5-8 may

account in part for the low incidence of mutations found. The subjects recruited into the study presented in the main with early breast cancer and were all treated with primary surgery. Patients with large or locally advanced tumours who received neoadjuvant systemic therapy with either chemotherapy or endocrine therapy prior to surgery were excluded from the study. Given the high relative frequency of p53 mutations in locally advanced breast cancers (Faille et al 1994) this is a potential bias that may have affected the incidence of mutations found in the current study. The study population was restricted to women aged 55 or younger and this is another potential bias.

It proved impossible to demonstrate p53 abnormalities in the nipple aspirate fluid samples of the subjects whose breast tumour specimens were shown to carry mutations. Indeed it was only possible to obtain a PCR product from 2 out of the 4 samples, neither of which demonstrated an abnormal band on SSCP analysis. It is known that a proportion of nipple aspirate samples will be either acellular or sparsely cellular, and this may account for the failure to obtain a PCR product from the 2 fluid samples concerned. It appears that breast fluid samples contain an inhibitor to PCR reactions when using the conditions employed in this study. The addition of a small quantity of NAF (5µl or less) to a 100µl PCR reaction containing 100ng of extracted cell line DNA resulted in either the complete inhibition of the reaction or a significant reduction in the quantity of product obtained. It was not possible to undertake studies to define the nature of the factor or factors responsible. However it was concluded that for the PCR conditions used it was necessary to extract DNA from the sample fluid rather than simply release DNA into the PCR

reaction solution by cell lysis. Out of necessity this will result in a dilution of DNA concentration and possibly loss of some material in the various steps involved both of which may be of critical importance when samples contain very small amounts of genetic material. DNA in sufficient quantities to subject to PCR could be obtained from approximately half the fluids tested using a modification of a commercially available DNA extraction kit. It would obviously be advantageous to improve upon this ratio, and the development of a method analogous to that used for single cell sample such as sperm (Li et al 1988) would be a significant advance in allowing the study the molecular biology of nipple aspirate fluids.

The reasons for the 2 successfully amplified nipple aspirate samples not demonstrating any evidence of the p53 mutation detected in the corresponding tumour sample bear exploration. An obvious explanation could be that there was no shed tumour material in the fluid. It is noteworthy that Sartorius et al (1977) have reported an inverse correlation between tumour size and the likelihood of nipple aspirate fluid demonstrating cytologically abnormal cells. They argue that the larger the tumour the more likely it is that the ductal architecture of the diseased part of the breast is disrupted and consequently the fluid aspirated coming from a healthy part of the breast, citing evidence from radiological studies confirming such distortion. However evidence from a detailed pathological study of mastectomy specimens (Andersen and Pallesen 1979) demonstrated the presence of tumour within 10mm of the nipple in 50% of specimens studied, and whilst this could be accounted for as much by tumour multifocality as by intra-luminal spread it does suggest that using optimal techniques a proportion of nipple aspirate fluids may reasonably be expected to contain shed tumour DNA either in

the form of intact cells or cellular debris. Another potential explanation is that the nipple aspirate samples did contain tumour derived DNA, but in low proportion relative to that of shed non-tumour cells. As was demonstrated in this thesis SSCP was able to detect between 1 and 30 mutation bearing cells per 100 in cell line mixing experiments, and thus if tumour cells comprised a lesser amount of the sample their presence would not be detected by SSCP. Mutant specific primers were developed for one of the mutations found but using these it was not possible to detect presence of the mutation in the corresponding NAF sample. Unfortunately it was not possible to assess formally the sensitivity of this system to pick up small numbers of mutant carrying cells amongst larger numbers of wild type due to lack of a pure source of mutant DNA to allow quantification. However when mutant specific primers have been used in other situations they have been reported to detect 1 mutant cell in 10^{5-6} (Cha et al 1992, Jacobson and Mills 1994, Rhodes et al 1997). It is therefore likely that the nipple aspirate sample from the subject in question carried little or no tumour derived DNA.

A further limiting factor was the small quantity of fluid obtained from some subjects and the difficulty in efficiently collecting it. The fluid appeared subjectively to vary in viscosity between subjects and collection of fluid by capillary action into glass tubes worked well only for a proportion of subjects, in others whilst fluid could be collected there was significant wastage of scant material. In the latter part of the study fluid was aspirated from the nipple surface using a 200 μ l micropipette. This was technically easier and less wasteful of precious sample and would be recommended as the method of choice for future studies.

The quantity of fluid obtained was variable and small. It was not possible to determine whether the collection of aspirate samples with the patient anaesthetised had any adverse bearing on yield though in theory this is possible. Previous authors have emphasised the importance of the patient gently massaging the breast during sample collection (Papanicolaou et al 1958, Sartorius et al 1977) and psychological factors such as the patient being in a relaxed and comfortable state during the procedure appear to be important predictors of success (I. McFadyen personal communication of unpublished observations). Reasons for the procedure being carried out under anaesthetic were discussed in Chapter 2.3.2.. The question arises as to whether or not the yield of fluid could in any way be enhanced. Galactorrhea is a potential side effect of a variety of drugs, mainly with dopamine antagonist activity (Thorner MO 1996), and administration of such an agent could theoretically enhance yield of nipple aspirate fluid samples. There are no published data investigating this possibility and whilst such an approach would not find routine use it may, if successful, have a role in well defined circumstances.

It is clear from the published data discussed in the introduction that useful information about the biochemistry and pathophysiology of breast cancer has been derived from the study of nipple aspirate fluids. This study aimed to take this branch of breast cancer research forward by investigating whether tumour specific genetic mutations could be detected in such fluid samples. That it failed to do so should not close the door on future attempts but rather attention should be turned to addressing the following issues:

- 1) The optimal collection and preparation of nipple aspirate fluid for genetic analysis, including assessment, if possible, of sample cellularity is

of great importance.

2) Consideration should be given to which subjects can most profitably be studied. Sartorius et al (1977) demonstrated a lower likelihood of abnormal cytology in nipple aspirates from subjects with large breast cancers. It may be appropriate therefore to concentrate further efforts on those with small lesions or with carcinoma in situ, groups less likely to have disruption of ductal architecture, but it has to be remembered that to evaluate studies on mutation detection it is desirable to have tissue from an operative specimen to confirm the presence in the lesion of any mutations found in the corresponding fluid. Were such methods found to be valuable extending their use to the study of high risk groups such as found in breast cancer family clinics, may be appropriate, not only from a scientific point of view but also from a clinical one.

3) Attention is required to which marker or markers should be examined. As described in the introduction p53 mutations are probably the most common genetic aberrations reported to date in breast cancer. The abnormalities have the additional advantage of being qualitative rather than quantitative, which would tend potentially to increase sensitivity. However considerably over half of all breast cancers do not have a p53 mutation, and therefore even if a reliable method for detecting such mutations in nipple aspirate samples were developed its scope for use as an adjunct to screening programmes would be limited to a subset of patients. Recent data in preliminary form may however be relevant (Crook et al 1997). All 8 tumours (7 breast and 1 ovarian cancers) from 4 different families carrying BRCA-1 germline mutations were found to have p53 mutations. The authors conclude that acquired p53 mutations may have a pivotal role in the development of BRCA-1 associated cancers. The sequential study of nipple aspirate samples from both breasts

in women from such families may be potentially a useful adjunct to the mammographic and clinical surveillance already offered to them, particularly when the difficulties of interpreting premenopausal women's mammograms are considered (screening, in this context, often starting many years before the menopause). The physiology of the BRCA-1 protein is by no means established but the description of it as a secreted molecule present in breast milk is potentially of interest in future studies (Jensen et al 1996, Diamandis 1996).

As a greater understanding of the molecular biology of sporadic breast cancer unfolds other potential markers for such studies may become apparent.

4) What is the most appropriate method for mutation detection? This would very much depend on the gene of interest. For instance a gene like p53 in which mutations are described throughout a large part of the gene requires a screening method such as SSCP to potentially detect the majority of mutations. In colonic and pancreatic cancers mutations in the RAS family of genes are common, and because most mutations are confined to a few codons it is feasible to use mutation specific approaches to enhance sensitivity in detecting mutations in the background of a large proportion of normal cells. However RAS mutations are uncommon in breast cancer (Capella et al 1991)

5) Is there scope for investigating methods other than mutation detection? In good quality cellular aspirates immunohistochemical analysis may have a role, with the relative expression of various oncogene products being inferred. The limiting factor here being the cellularity of the sample. However with the recent description of a method by which a single paraffin embedded tissue slice was used for both immunohistochemical and subsequent DNA analysis (Chen et al

1996), it is feasible that such an approach could be extended to a nipple aspirate smear on a microscope slide.

In conclusion it remains to be hoped that, with the rapidly ongoing developments in molecular biological techniques, studies on genetic abnormalities in nipple aspirate fluid samples may further the understanding of the biology of breast cancer and potentially find a clinical role.

CONCLUSIONS

Mutations of the p53 gene are a common feature of a variety of malignant diseases. The studies set out in this thesis investigated the potential for using the detection of p53 mutations as a means of demonstrating the presence of malignancy in 2 biological fluids, namely in nipple aspirate fluid samples from patients with breast cancer and in bladder washing samples from patients with bladder cancer.

SSCP was the method used to screen samples for the presence of mutations. Before its use on clinical samples, the methodology was optimised and its sensitivity evaluated using cell lines with known p53 status. Using polyacrylamide gel electrophoresis it was demonstrated that an abnormal SSCP pattern was discernible under optimal conditions for samples in which mutation-carrying cells represented as little as 5% of the total. Optimal conditions varied between particular mutations studied and were not predictable; thus to maximise the sensitivity of the technique to detect mutation carrying cells in the background of an excess of wild type cells it was necessary to use 2 or more sets of electrophoresis conditions in a largely empirical manner. The use of MDE as a gel matrix for SSCP was shown to offer no advantage over polyacrylamide.

The ductal epithelium of the breast is the site of origin of the majority of breast cancers. Breast ductal fluid is secreted by this epithelium and samples of the fluid obtained by nipple aspiration from patients with breast cancer can exhibit cellular and biochemical features reflecting the presence of the underlying disease. The detection of cancer-related gene mutations in nipple aspirate fluids has not to date been reported and the

current studies were unable to demonstrate p53 mutations in nipple aspirate fluids from patients whose primary tumours carried mutations. Problems encountered included the limits of sensitivity of the mutation detection assay used and the preparation of DNA from small samples for PCR. Concern remains that distortion of breast ductal architecture by invasive cancers may prevent, at least on occasions, the aspiration of fluid from the diseased ductal system. Further studies are however warranted and these issues would need to be addressed. The study of molecular abnormalities in nipple aspirate fluid samples could potentially further the understanding of the molecular events involved in breast carcinogenesis, and could eventually become clinically relevant to the disease screening of individuals at high-risk.

Patients with bladder cancer shed tumour cells into the bladder. Despite this, urine cytology and other non-invasive tests have well documented limitations with the result that the diagnosis and follow-up of patients with, or suspected of having bladder cancer rely heavily on invasive techniques. A variety of approaches have been adopted to enhance the diagnostic potential of urine and bladder washing samples. Of these the study of cancer-related molecular abnormalities is of particular interest and potential. The current study demonstrated that it was possible to detect p53 mutations in bladder washing specimens from a proportion of patients with bladder cancer. Furthermore it was possible to define the exact nature of the mutation in some cases and confirm the presence of the same abnormality in bladder tumour samples. There were occasions however when a mutation was present in tumour samples but not detectable in corresponding bladder washing samples. The most likely explanation being the limited sensitivity of the mutation detection assay

(SSCP) employed. This work and that of others enforce the potential for the use of molecular markers in bladder cancer. Further studies are clearly warranted both on bladder washing samples and on urine samples with issues of sensitivity and correlation with clinical and pathological findings being of paramount importance.

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Appendix 1

Patient information sheet and consent form for nipple aspirate subjects

PATIENT INFORMATION SHEET

We would like to explain some research being done by Dr Hamish Phillips, a Research Fellow at the University, and then ask you if you would like to take part.

We are studying the differences between normal cells and cancer cells in the laboratory. This usually involves taking a specimen during an operation but we are interested in assessing very small samples which could avoid the need for biopsies for this type of research.

You are about to have an operation to remove the tumour or cancer from your breast. We would like to take a small piece of the tumour to be studied in the laboratory and in addition will ask you to let us take a small sample of the fluid normally found in the breast to be studied in the same way. The fluid will be obtained by applying gentle suction to the nipple by means of a plastic cup placed over the nipple. This will be done whilst you are under a general anaesthetic for your operation.

Your treatment will be entirely unaffected by this study should you choose to take part.

No side-effects are expected from the collection of the fluid from your breast but the procedure may cause mild discomfort. The method has been widely used in the past.

You are under no obligation to take part in the study. If you do decide to take part you are free to withdraw or change your mind at any stage without any fear of this influencing your future treatment.

Mr U. Chetty

Professor W.R.Miller

CONSENT FORM

PROJECT TITLE:

AN ASSESSMENT OF ABNORMALITIES OF THE GENE P53 IN
BREAST AND BLADDER CANCERS USING EXFOLIATED (SHED)
TUMOUR MATERIAL

Name of Investigator: Dr. Hamish Phillips
Dept. of Clinical Oncology,
Western General Hospital ,
Edinburgh
031 537-1000

I agree to participate in this study

I have read this consent form and the attached Patient Information Sheet
and had the opportunity to ask questions about them

I agree for notice to be sent to my General Practitioner about my
participation in this study

I agree to the provision of any clinically significant information to my
General Practitioner

I understand that I am under no obligation to take part in this study and
that a decision not to participate will not alter the treatment that I would
normally receive

I understand that I have the right to withdraw from the study at any stage
and that to do so will not affect my treatment

I understand that this is non-therapeutic research from which I can't
expect to derive any benefit

Name of Patient:

Signature of Patient:

Signature of Investigator:

Date:

Appendix 2

Sequence of Exons 5, 6, 7 and 8 of the human p53 gene and surrounding intronic areas (sequence x54156 obtained from the European Molecular Biology Laboratory Genbank database: submitted by Chumakov PM). Exon sequences are in upper case and intron sequences in lower case, with underlined sequences representing sites of primers.

Exon 5: 5' tgtgccctgactttcaactc tgtctccttctcttctctacag TAC TCC CCT
GCC CTC AAC AAG ATG TTT TGC CAA CTG GCC AAG ACC TGC
CCT GTG CAG CTG TGG GTT GAT TCC ACA CCC CCG CCC GGC
ACC CGC GTC CGC GCC ATG GCC ATC TAC AAG CAG TCA CAG
CAC ATG ACG GAG GTT GTG AGG CGC TGC CCC CAC CAT GAG
CGC TGC TCA GAT AGC GAT G gtgagcagctggggctgg
agagacgacagggctggtt 3'

Exon 6: 5' tgattcctcactgattgctc ttag GT CTG GCC CCT CCT CAG
CAT CTT ATC CGA GTG GAA GGA AAT TTG CGT GTG GAG
TAT TTG GAT GAC AGA AAC ACT TTT CGA CAT AGT GTG
GTG GTG CCC TAT GAG CCG CCT GAG gtctggttgcaactgggg 3'

Exon 7: 5' aaggcgactggcctcatct tgggcctgtgttatctctag GTT GGC TCT
GAC TGT ACC ACC ATC CAC TAC AAC TAC ATG TGT AAC
AGT TCC TGC ATG GGC GGC ATG AAC CGG AGG CCC ATC CTC
ACC ATC ATC ACA CTG GAA GAC TCC AG gtcaggagcc
acttgccaccctgcacactgg 3'

Exon 8: 5' ggacctgatttcctttactgc ctcttgcttctctttctatctctgagtag GGT
AAT CTA CTG GGA CGG AAC AGC TTT GAG GTG CGT GTT TGT
GCC TGT CCT GGG AGA GAC CGG CGC ACA GAG GAA GAG
AAT CTC CGC AAG AAA GGG GAG CCT CAC CAC GAG CTG
CCC CCA GGG AGC ACT AAG CGA G gtaagcaagcaggacaagaagcgggtgga
ggagac caaggggtgcagttatgcctc 3'

Appendix 3

Appendix 3 tabulates on the following 2 pages the clinical details of subjects from whom bladder washing samples were obtained. Details include patients' age, whether or not fresh tumour was obtained at the same time as the bladder washing, the histological type, pathological stage and grade of the bladder cancer, as well as details of previous treatment received.

Appendix 3

CLINICAL DETAILS OF SUBJECTS FROM WHOM BLADDER WASHING SAMPLES WERE OBTAINED

Patient Code	Washing Number	Age	Tumour Available	Pathology	Stage	Grade	Previous Treatment
A	3	66	YES	TCC	pT2	G3	RADIODTHERAPY
B	6	86	YES	TCC	pT2	G3	NIL
C	7	55	YES	TCC	pTa	G2	NIL
D	8	80	YES	TCC	pTa	G2	RES: G1 pTa TCC
E	9	67	YES	TCC/SQ	pT2	G3	NIL
	24	67	YES	TCC/SQ	pT1x	G3	RADIODTHERAPY
F	10	52	NO	TCC	pTa	G1	RES: G2 pTa TCC
G	12	83	YES	TCC	pT2	G3	NIL
	33	84	YES	TCC	pT2	G3	RADIODTHERAPY
H	13	82	YES	TCC	pT2	G3	RADIODTHERAPY
I	14	63	YES	TCC	pT2	G3	RES: G2 pT2 TCC
J	15	82	YES	TCC	pT2	G3	RADIODTHERAPY
	31	82	NO		REC NO Bx		RADIODTHERAPY
K	16	66	YES	SQ	pT2	G3	RADIODTHERAPY
L	17	63	NO	TCC	pT1	G3	RADIODTHERAPY
M	18	78	YES	TCC	pT1/x	G3	NIL
	26	78	YES	TCC	pTa	G3	IVESC: G3 pT1/x TCC
N	19	76	YES	TCC	pT2	G3	NIL
O	20	60	YES	TCC	pT1	G2	NIL
P	21	76	NO	*CIS	N/A	N/A	RES, IVESC: G3 pT1 TCC

Appendix 3 Continued

Patient Code	Washing	Number	Age	Tumour	Available	Pathology	Stage	Grade	Previous Treatment
Q		22	75	YES		TCC	pTa	G3	RES, IVESC: G2 pT1 TCC
R		23	77	NO		TCC	pT2	G3	NIL
S		25	86	YES		TCC	pTx	G2	NIL
T		27	62	YES		TCC	pT1	G3	NIL
U		28	49	YES		TCC	pTa	G1	NIL
V		29	51	NO		TCC	pT2	G3	NIL
W		30	41	YES		TCC	pT2	G3	NIL
X		32	62	NO		*DYSPLASIA			RES: G1 pTa TCC
Y		40	51	YES		TCC	pTa	G1	NIL
Z		41	63	YES		TCC	pT2	G3	NIL
AA		50	57	NO		TCC	pTa	G1	RES: G1 pTa TCC

KEY:

TCC

SQ

TCC/SQ

RES

RADIO THERAPY

IVESC

G 1-3

pT

* CIS

* DYSPLASIA

REC NO Bx

Transitional Cell Carcinoma

Squamous Carcinoma

Mixed Transitional and Squamous Carcinoma

Transurethral Resection of Tumour

Radical Radiotherapy

Intra-Vesical Chemotherapy

Histological Grade (G1: well, G2: moderately and G3: poorly differentiated)

Pathological stage pTa: no invasion of lamina propria, pT1: invades lamina propria,

pT2 invasion of detrusor muscle, pTx: invasion can't be assessed, pT1x: at least pT1

Visible abnormality at cystoscopy, biopsy revealed carcinoma in situ

Visible abnormality at cystoscopy, biopsy revealed dysplasia only.

Recurrent tumour seen at cystoscopy: no biopsy or resection performed

Appendix 4

Appendix 4 tabulates the clinical details of the subjects from whom nipple aspirate fluid samples were obtained. Details include patients' age, the size of their tumour, the nature of their operation, the pathological type, grade and oestrogen receptor status of the tumours and finally whether or not a sample of macroscopically normal breast tissue was obtained.

Key:

Size	Clinically measured tumour size
0	0
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9
10	10
11	11
12	12
13	13
14	14
15	15
16	16
17	17
18	18
19	19
20	20
21	21
22	22
23	23
24	24
25	25
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78	78
79	79
80	80
81	81
82	82
83	83
84	84
85	85
86	86
87	87
88	88
89	89
90	90
91	91
92	92
93	93
94	94
95	95
96	96
97	97
98	98
99	99
100	100

Operation MX : Mastectomy

WLE: Wide Local Excision

R : Right

L: Left

Pathology ID NST : Invasive Ductal Carcinoma of No Special Type

ILOB: Invasive Lobular Carcinoma

I CRIB: Invasive Cribriform

DCIS : Ductal Carcinoma in Situ

Grade	Histological Grade for Invasive Cancers
1	Grade 1
2	Grade 2
3	Grade 3

1: Well differentiated

2: Moderately differentiated

3: Poorly differentiated

ER Status Oestrogen Receptor Status

-VE Oestrogen receptor poor or negative. Level >20 fmol per mg protein being regarded as ER rich.

+ VE Oestrogen receptor rich

Appendix 4

Patient Code	Tumour Number	Age	Size	Operation	Pathology	Grade	ER Status	Normal Breast Sample
A	1	45	1.9 cm	R WLE	ID NST	2	+VE	NO
B	2	41	1.0 cm	L MX	ID NST	3	+VE	NO
	3		1.5 cm		ID NST	3	+VE	NO
	4		3.0 cm		ID NST	3	+VE	NO
C	5	49	2.7 cm	L MX	DCIS	HIGH	-VE	NO
D	6	34	4.6 cm	R WLE	ID NST	3	-VE	YES
E	8	51	2.0 cm	L WLE	ID NST	2	+VE	NO
F	9	55	1.5 cm	L WLE	ID NST	3	+VE	NO
G	10	49	2.6 cm	R MX	ILOB	2	+VE	YES
H	12	40	4.0 cm	R WLE	ID NST	1	+VE	NO
I	13	47	3.1 cm	L WLE	ILOB	2	+VE	NO
J	14	48	1.9 cm	L WLE	ID NST	2	+VE	NO
K	15	45	1.5 cm	L WLE	ID NST/ILOB	2	+VE	NO
L	16	34	4.2 cm	R WLE	ID NST	2	+VE	NO
M	17	54	2.0 cm	R WLE	ILOB	2	+VE	NO
N	18	47	2.0 cm	R WLE	ID NST	3	-VE	YES
O	20	50	3.0 cm	R MX	DCIS	HIGH	-VE	NO
P	21	50	4.0 cm	R MX	ID NST	2	+VE	YES
Q	23	52	5.0 cm	L MX	ID NST	3	-VE	YES
R	25	47	2.5 cm	R WLE	ID NST	3	-VE	NO
S	26	51	1.5 cm	L WLE	I CRIB	1	+VE	NO
T	27	45	1.0 cm	R WLE	ID NST	2	+VE	NO
U	28	39	2.8 cm	R WLE	ID NST	2	+VE	NO